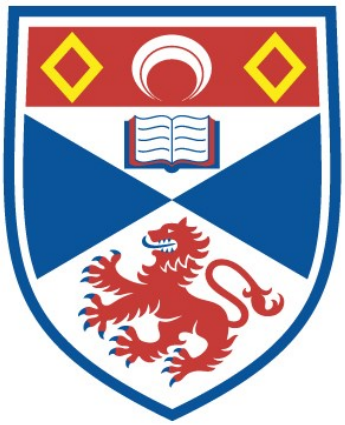
AN EXAMINATION OF THE LIPID-SOLUBLE COMPONENTS OF PLANT LEAVES WITH PARTICULAR REFERENCE TO THE PIGMENTS AND QUINONES PRESENT

Colin Peter Bond

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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AN EXAMINATION OF THE LIPID-SOLUBLE COMPONENTS OF PLANT LEAVES, WITH PARTICULAR REFERENCE TO THE PIGMENTS AND QUINONES PRESENT.

by

COLIN PETER BOND, B.Sc.

A thesis

presented to the University of St. Andrews for the Degree of Doctor of Philosophy.

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry in the United College of St.Salvator and St. Leonard, St. Andrews, under the supervision of Professor G. R. Tristram.

C E R T I F I C A T E

I hereby certify that COLIN PETER BOND has spent nine terms engaged on research work under my supervision, and that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I first matriculated at the University of St. Andrews in September, 1961, and studied the subjects of Biochemistry, Botany and Chemistry at the Second Science level. I was admitted into the Honours Class in Biochemistry in October, 1963, obtained an Upper Second Class Honours Degree in Biochemistry in June, 1965 and graduated in July of that year. I was then admitted as a research student to the Department of Biochemistry in United College in August, 1965. Since that time, I have been engaged in research, the results of which are now submitted for the Degree of Doctor of Philosophy of the University of St. Andrews. I have been supported throughout the major part of this work by a research grant from British Glues and Chemicals Limited and, during the summer of 1967, by a postgraduate scholarship from the University of St. Andrews.

A C K N O W L E D G E M E N T S

I would like to express my appreciation to Professor Tristram for his helpful supervision and guidance which made this work possible.

I am indebted to British Glues and Chemicals Limited for financial support during the period 1st of August, 1965 to 31st of July, 1967 and to the Postgraduate Awards Committee of the Senate of the University of St. Andrews for the period 1st of August to 30th of September, 1967.

I would also like to thank Dr. D. Thirkell for helpful advice and criticism, Mr. D. Wishart for assistance with the computer studies, and the staff of the University Botanic Gardens for growing the leaf material used during this work. I would also like to acknowledge the technical services provided by the Biochemistry Department and to thank Mr. W. B. Blyth for the photography.

Finally, I would like to express my gratitude to my wife for her encouragement throughout this work, and to thank her for typing this finished article.

C O N T E N T S

EXPERIMENTAL SECTION (contd.)

RESULTS SECTION

RESULTS SECTION (cont'd.)

INTRODUCTION

INTRODUCTION

When the leaves of higher plants are macerated with various organic solvents, the extracts obtained are complex mixtures of lipids and lipid soluble materials. Bloor (1943) considers lipids as "a group of naturally occurring substances consisting of the higher fatty acids, their naturally occurring compounds, and substances found naturally in chemical association with them", but the term lipid is often used more loosely to include the substances extracted together with lipids by treatment of biological material with organic solvents. In this discussion and the work that follows, the term lipid is used in its looser sense in order to include such aliphatic hydro-carbons, terpenoids and their derivatives as are co-extracted with the true lipids.

The first components of the leaf-lipid extracts to be examined were the pigments; and as long ago as 1860 Frémy was able to show that more than one pigment was present in such extracts. In actual fact, Frémy (1860) isolated an ether extract of the yellow carotenoids, which he termed "phylloxanthin" and an aqueous acid extract of blue-green chlorophyll derivatives (phaeophytins and phaeophorbides) which he termed "phyllocyanin".

Four years later Stokes (1864a, 1864b), an English spectroscopist, showed, by fluorescence and solvent partition studies, that two different green pigments were

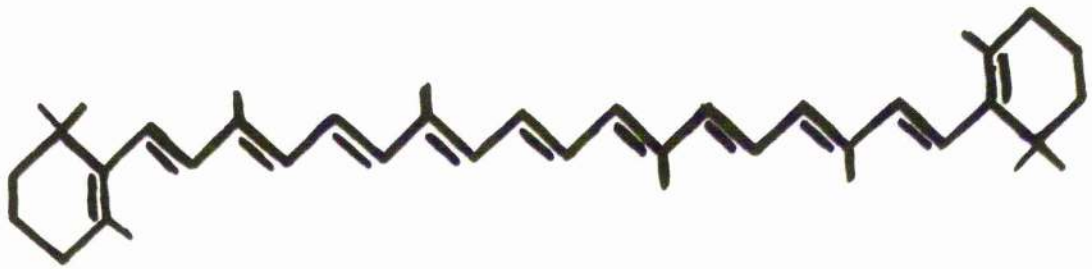
present as well as two yellow compounds (actually a mixture of several carotenoids). Sorby (1873) extended Stokes' work and found a third chlorophyll which he termed chlorofucine (now known as chlorophyll c) to be present in the brown algae. By differential solubility and partition methods, Sorby was also able to show the presence of several "xanthophylls" in plant leaves, three of which can be recognised, as carotene ("orange xanthophyll"), lutein ("xanthophyll") and violaxanthin ("yellow xanthophyll"). It was not until 1906 however, that a satisfactory separation of the pigments was achieved, by Tswett, using his method of "chromatographic adsorption analysis" on finely divided materials such as calcium carbonate. Tswett (1906) found, in his extracts of green leaf material, an orange pigment (carotene), two green chlorophylls and three or four yellow xanthophylls, but Willstätter and Mieg (1907) found only one xanthophyll to be present, which they isolated in crystalline form. Willstätter and Stoll (1913) suggested that the xanthophylls found by Tswett might have been alteration or oxidation products of the single pigment found by their own team, whereas Tswett contended that they had isolated only the xanthophyll which he had found in greatest concentration.

Palmer (1922) repeated Tswett's work and confirmed the Russian botanist's original results, but controversy continued until Strain's classic work between 1934 and 1937 (Strain, 1938a). Strain used column chromatography, mainly

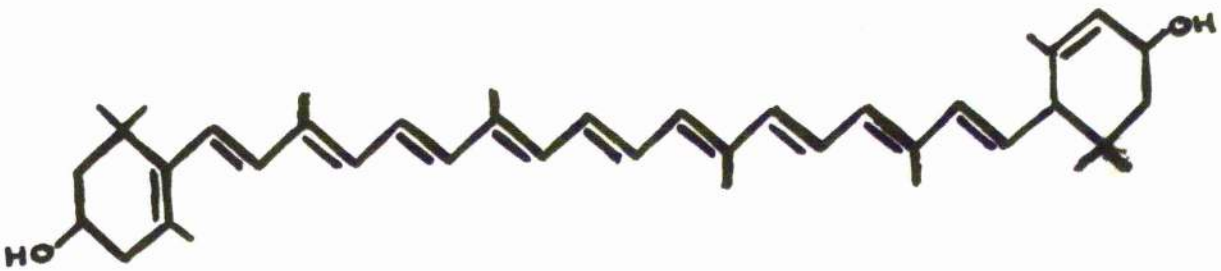
on magnesia, to separate the carotenoid pigments present in saponified leaf extracts and he concluded that two carotenes (α and β carotenes) were usually present, together with at least twelve xanthophylls. It was later realised that some of the pigments found by Strain were artefacts produced by the action of HCl (formed by the breakdown of the solvent dichloroethane), and in reappraisal of his own work, Strain (1948b) concluded that only four xanthophylls were present in green leaves (lutein, neoxanthin, violaxanthin and zeaxanthin) together with traces of another (cryptoxanthin). Since then, various workers have separated the carotenoids of leaves by column chromatography with fairly similar results to Strain's: Moster, Quakenbush and Porter (1952); Bickoff, Livingston, Bailey and Thompson (1954); Cholnoky, Györgyfy, Nagy and Pánczel (1956); Friend and Nakayama (1959); Yamamoto, Chichester and Nakayama (1962a). The structures of the four common leaf carotenoids are shown in fig. 1 and those of the four others sometimes reported, in fig. 2.

Willstäter and Stoll (1913) confirmed Tswett's original observations that there were two chlorophylls ("a" and "b") present in leaves and found that these were in a fairly constant ratio. Mackinney (1940, 1941) and Comar and Zscheile (1941, 1942) confirmed the findings of Willstäter and Stoll, and presented spectrophotometric methods for the analysis of plant extracts for chlorophylls a and b. Since then, various workers have presented methods for the spectro-

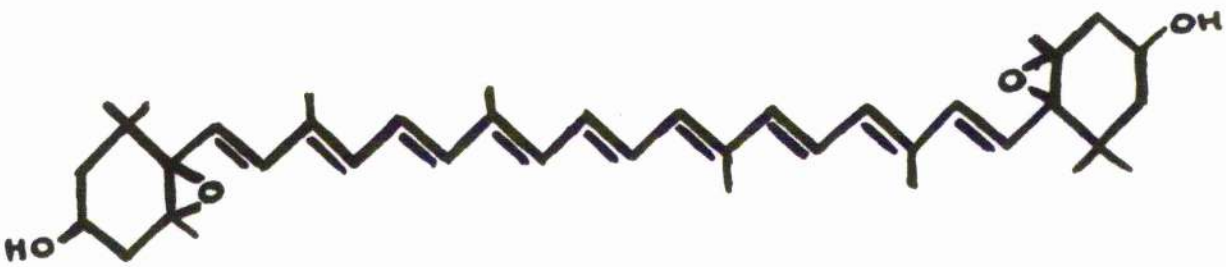
MAJOR CAROTENOIDS OF LEAVES



β-carotene



Lutein



Violaxanthin

Neoxanthin

MINOR CAROTENOIDS OF LEAVES

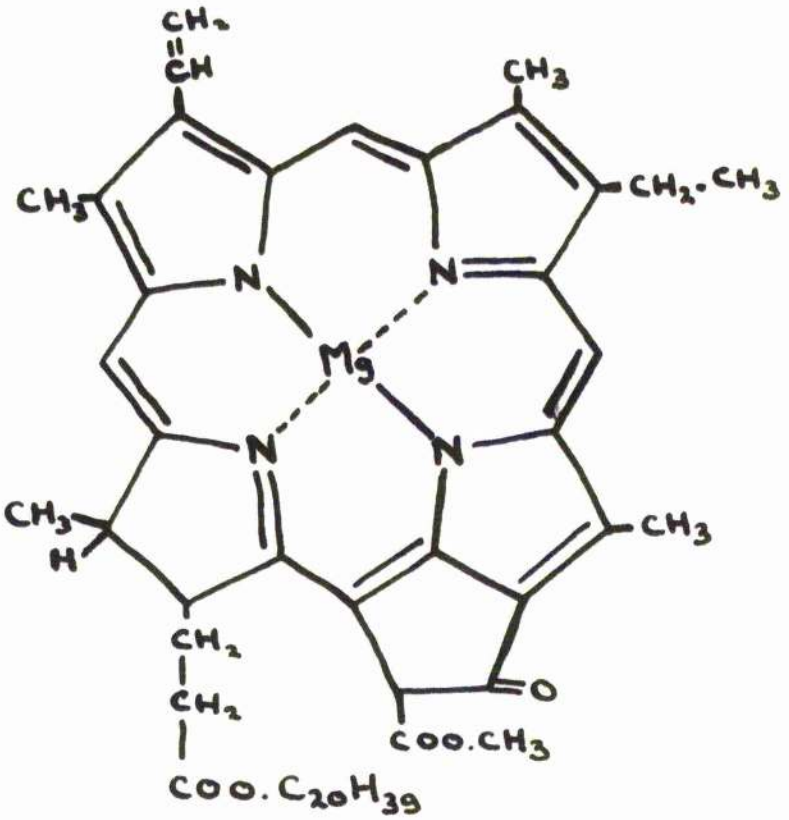
α-carotene

β-cryptoxanthin

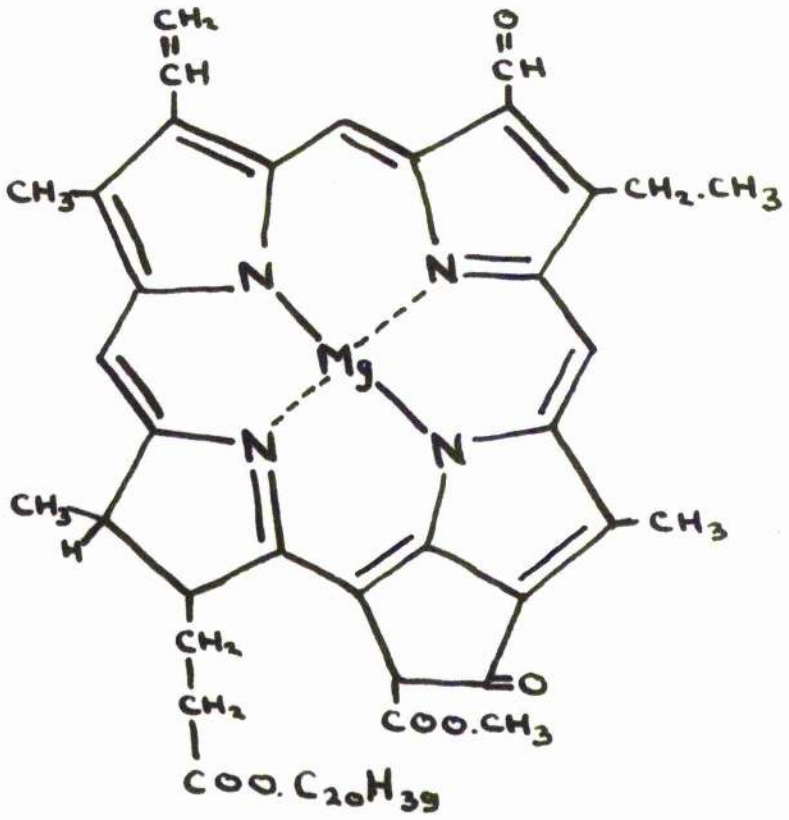
α-cryptoxanthin

Antheraxanthin

Fig. 3



Chlorophyll a



Chlorophyll b

photometric determination of chlorophylls in leaf extracts using various organic solvents. Hoffman and Werner (1966) in a critical review of these methods suggest that three are particularly reliable; those of Holm (1954) using 100% acetone, and those of Comar and Zscheile (1942) and Smith and Benitez (1955) using diethyl ether as the solvent. The method of Ogawa and Shibata (1965) is also useful, particularly for the determination of small amounts of chlorophyll b. This method relies on the conversion of the aldehyde group in chlorophyll b to the oxime form, by means of hydroxylamine hydrochloride; from the absorption change at a single wavelength (666nm) the concentrations of chlorophylls a and b can be calculated. The structures of chlorophylls a and b are shown in fig. 3.

In the hope of achieving more rapid separations, many workers have attempted to separate the lipid soluble plant pigments by means of paper chromatography. A review of over 50 papers on this subject is presented by Sestak (1959) and another review of a further 150 or so by Sestak (1965). The majority of these workers have used normal chromatography paper, but adsorbent-impregnated or "filled" papers have been used satisfactorily by Angapindu, Silberman, Tantivatana and Kaplan (1958), Jensen and Jensen (1959) and Jensen and Aasmundred (1963). Booth (1962) has achieved good separation of the lipid components of leaves, including some of the pigments, with a two dimensional technique using adsorbent ($ZnCO_3$)-impregnated paper in the first direction

and impregnation with paraffin to give a "reversed-phase" system for the second dimension.

Apart from a report by Anderson (1960) using centrifugally accelerated paper chromatography (which is rather complex), the methods available for the separation of the chlorophylls and carotenoids of plant extracts are somewhat time consuming and, since the pigments are very labile, this can prove a great disadvantage. The best results have been achieved with adsorption chromatography and since thin layer chromatography (TLC) according to Stahl (1956), (although it had previously been used, e.g. Kirchner, Miller and Keller, 1951) is a micro-method of adsorption chromatography, it seemed that it should be possible to obtain separations of the lipid-soluble plant pigments using this technique. Prior to 1964 the only reports of the application of TLC to the separation of chlorophylls and carotenoids were those of Egger (1962), using a reversed-phase technique, Hager and Berentrath (1962) who used partition chromatography on a mixed support, and Grob, Eichenberger and Pflugshaupt (1961) who used adsorption chromatography on Silica Gel. The last report however, gives chromatograms that appear very uneven and are suggestive of poor reproducibility. It was thus decided that an investigation into the use of TLC for the separation of plant pigments might produce a rapid method of analysis which could be used for the study of variations in leaf

carotenoids under different physiological and environmental conditions. Since 1964, several workers have described the TLC of leaf pigments using various different supports. Bunt (1964) has described the separation of algal pigments on Kieselguhr layers; Bacon (1965, 1966) and Schneider (1966) have described the TLC of various leaf pigments on cellulose layers; Colman and Vishniac (1964) and Smith, Breidenbach and Rubenstein (1965) have utilised thin layers of various sugars, whilst Rai and Lee (1964) have separated algal pigments by TLC on mixed layers of cellulose and sucrose.

Silica Gel layers incorporating some liquid paraffin have been used by Schaltegger (1965) for the TLC of chlorophylls and carotenoids, whilst the use of normal Silica Gel layers has been reported by Seliskar (1966), Lynn and Schanderl (1967) and Bond (1967), the last report being a part of the results of this present work.

Microscopic examination of plant leaves shows that the leaf pigments are located in small bodies known as chloroplasts. The chloroplasts are the site of photosynthesis in the plant cell - Arnon, Whatley and Allen (1954), and Hill (1937) - and they have a similar structure in all higher plants. The typical higher plant chloroplast is lens shaped when seen at right angles to its plane, may be circular or elliptical in outline (Kirk and Tilney- Basset, 1967) and between 3 and 10 microns across (Möbius, 1920).

Heitz (1936) was able to show that the chlorophyll in the chloroplasts was not uniformly distributed but concentrated in small particles that he termed grana. Heitz considered that the grana were dispersed in a matrix, which he termed the stroma, which was itself contained by the bounding membrane of the chloroplast (now shown to be a double membrane - Heslop-Harrison, 1963). Electron microscopy has shown that these grana are composed of stacks of disc-like membrane-bounded sacs which Menke (1962) has called thylakoids. These membranes form what is known as the lamellar system of the chloroplast and they extend from the thylakoids into the stroma of the chloroplast to form the stroma lamellae, which interconnect with other grana of the chloroplast. Electron micrographs frequently show complex spatial relationships at the margins of the grana (see Weier and Thomson, 1962) which indicate that each stroma lamella may interconnect with several grana thylakoids. This has led Heslop-Harrison (1963, 1964, 1966) to suggest that there may be only two volumes in the chloroplast, the stroma and the "unimaginably complex intra-thylakoid space". Wehrmeyer (1964) has suggested a somewhat similar model for the inter-relationships of the chloroplast lamellar system. Park and Pon (1961) isolated the lamellae from sonically irradiated chloroplasts and showed that the light reactions of photosynthesis were localized solely within the lamellar fraction; the dark reactions and the enzymes of

carbon dioxide fixation were found to be associated with the stroma portion of the chloroplast. Park and Pon (1961) were also able to show that the chlorophyll of the chloroplast was confined to the lamellar fraction.

Steinmann (1952) and Frey-Wyssling and Steinmann (1953) observed a granular structure of chloroplast lamellae, and Park and Pon (1961) suggested that the particles observed in metal shadowed electron microscope preparations were sub-units of the lamellar membrane and termed them quantasomes. Further work from the same laboratory (Lichtenthaler and Park, 1963; Park, 1965; Park and Biggins, 1964; Park and Pon, 1963) has shown the quantasome to be a particle approximately 180 x 150 x 100 Å in size, with a molecular weight of about 2×10^6 and containing about 230 chlorophyll molecules. The quantasome itself consists of 4 "90 Å" sub-units (Park, 1965) somewhat similar in size to the particles observed by other workers in mitochondrial and other cellular membranes e.g. Sjöstrand (1963), Fernandez-Moran, Oda, Blair and Green (1964), Green, Allmann, Bachmann, Baum, Kopaczyk, Korman, Lipton, MacLennan, McConnell, Perdue, Rieske and Tzagoloff (1967). It was originally thought that the quantasomes projected into the intra-thylakoid space and thus were on the inside of membrane but recent work by Park (1965) suggests that they may be on the outside of the thylakoids with single 90 Å sub-units only on the inside. This agrees, to some extent, with the results of Mühlethaler (1966) and Mühlethaler, Moor and Szarkowski (1965) from

work on freeze-etched preparations, although these workers found the sub-units to be only 60 Å in diameter.

Lichtenthaler and Calvin (1964) isolated whole chloroplasts and quantasome aggregates (lamellae) from Spinach leaves and determined the ratio of chlorophylls to carotenoids in each. Since these ratios were only slightly higher for quantasome aggregates, these workers concluded that the majority, if not all of the carotenoids in the chloroplast, were associated with the lamellar preparation. Park and Pon (1961) had already shown the association of chlorophyll with this fraction. Thus it is probable that, under certain conditions at least, the chloroplast pigments are to be found almost wholly associated with the lamellae and not in the stroma.

The role of chlorophyll in the quantum conversion of light energy into chemical energy during photosynthesis is well known. The actual mechanisms of photosynthesis are much more obscure and a great deal of research is at present in progress in this field. Several roles have been suggested for the carotenoid pigments of the chloroplast, the most important are probably those of accessory pigments in photosynthesis and protection against photodynamic damage. Action spectra for photosynthesis show that the so-called accessory pigments (mainly the carotenoids and chlorophyll b in higher plants) as well as chlorophyll a, can absorb light energy for photosynthesis. These accessory pigments are

presumed to pass their energy on to the reaction centres of the two pigment systems of photosynthesis, System I and System II, described by Duysens, Amesz and Kamp (1961).

Chlorophyll a in the chloroplast has been shown to exist in several different forms, although only a single homogeneous type of chlorophyll a is extracted by solvent treatment. The findings of Michel-Wolwertz and Sironval (1965) and Sironval, Michel-Wolwertz and Madsen (1965) that different forms of chlorophyll a can be extracted by solvents must be treated with caution in view of the findings of Bacon (1966), Bacon and Holden (1967) and of some of this present work. The number of different forms of chlorophyll a found in leaves varies with different reports, and although Thomas (1962) describes the presence of about seven different absorption bands, Cederstrand, Rabinowitch and Govindjee (1966), using an integrating spectrophotometer coupled with a computer, suggest that in Chlorella at least, there are only two bands, with absorption maxima at 668 and 683nm. A band at 695nm has often been reported (see French, 1966) in addition to the bands around 670 and 683nm. Kok (1961) managed to show the presence of a minor form of chlorophyll a (about 1/300 of the total chlorophyll a) which underwent light-induced bleaching. Since the absorption maxima of this bleaching was around 703nm, Kok termed this photochemically active form of chlorophyll, P700, and it is assumed to be the reaction centre of System I.

System II of Duysens and Amesz (1962) is considered to consist of chlorophyll a-683 (French, 1966), whilst System II refers to the shorter wavelength pigments, the carotenoids, chlorophyll b and chlorophyll a-670. As Duysens (1964) suggests however, these may only be the major components of each system and each pigment may be present in either system. In such a case, only the relative proportions of the different pigments may vary between the two systems. System I provides the reducing potential for the reduction of carbon dioxide, and System II, the oxidising potential for oxygen evolution.

The primary absorption of light by accessory pigment causes such pigments to be converted to singlet excited states (Clayton, 1966) and this singlet excitation energy is channelled by "inductive resonance" to the reaction centre of one of the two pigment systems; Livingston,(1960) suggests that the formation of addition compounds must also play a part in this energy transfer. It is possible that some of the energy transferred by either direct light absorption or by inductive resonance may convert a few of the chlorophyll molecules to an excited triplet state, and evidence for such a state in illuminated chlorophyll solutions has been observed (Linschitz and Sarkanen, 1958). Livingston (1960) has shown that carotene can quench such triplet states of chlorophyll and such quenching may be part of the mechanism of the protective action of carotenoids

in vivo (the energy of the triplet state may cause damage to the chlorophyll and perhaps to other surrounding materials). Krinsky (1966) discusses the protective action of carotenoids against lethal photosensitized oxidations in green plants, and suggests that zeaxanthin may deactivate complexes between chlorophyll and oxygen, by an epoxidation reaction resulting in its conversion to antheraxanthin.

The occurrence of a single compound, chlorophyll a, in several different forms in plant leaves is probably due to either aggregation of the chlorophyll molecules (see Brody and Brody, 1963) or interaction with some other substance such as protein. Earlier pigment protein complexes isolated by the method of Takashima, 1952 (eg. Chiba, 1955) appear to be either artefacts or merely physical associations (Anderson, Spikes and Lumry, 1954; Thirkell, 1964; Bailey, Thornber and Whyborn, 1966), but evidence for the existence of chlorophyll protein complexes in vivo is provided by Kahn and his co-workers who have extracted soluble pigment-protein complexes by treatment of chloroplasts with the non-ionic detergent Triton X-100 (Kahn, 1963, 1964; Kahn and Bannister, 1965; Kahn and Chang, 1965) followed by chromatography on DEAE- cellulose. Kahn (1963) reports that only 3-8% of the total chloroplast chlorophyll is contained in this complex.

Chiba (1960), Ogawa, Obata and Shibata (1966), Bailey,

Thornber and Whyborn (1966) and Thornber, Gregory, Smith and Bailey (1967) each report the isolation of two pigment protein complexes by the electrophoresis of anionic detergent-treated chloroplasts and chloroplast lamellae. Ogawa et al report that 60% of the chloroplast chlorophyll is present in their two protein complexes, whilst Thornber et al report around 75% of the lamellar protein (and thus 75% of the chlorophyll in their preparations) to be associated with the two protein complexes. The pair of pigment-protein complexes isolated by each of these teams of workers seem to be roughly equivalent, although there are differences in sedimentation co-efficients and in the ratio of chlorophyll a to b. It also seems probable from a comparison with work by Anderson and Boardman (1966) on digitonin-fractioned chloroplasts, that complex I from the gel electrophoresis studies is that responsible for the operation of System I of photosynthesis and complex II for System II. Further evidence for this is provided by the low values for chlorophyll b found in complex I, together with the earlier evidence for the association of chlorophyll b with System II. It is also interesting that Ogawa et al find the xanthophylls violaxanthin and neoxanthin (with epoxide oxygen) present in complex II alone, in view of the fact that System II is concerned in the evolution of oxygen during photosynthesis: Saakov (1964) has suggested that epoxy carotenoids may function in oxygen transfer during photosynthesis.

Further evidence for the existance of protein chlorophyll complexes in vivo is given by the work of Thomas and Bartels (1966) on the treatment of chloroplast preparations with aqueous acetone of different concentrations. Faludi-Dániel, Nagy, Gyurján and Faludi (1965) also produced evidence of chlorophyll protein complexes on the grounds that 95% of the chlorophyll was not extracted when chloroplasts were treated with petrol ether. After heat treatment however, only 50% of the chlorophyll remains after petrol ether extraction. These workers also found that the extractability of the carotenoids present increased from 66% to 80% on heat treatment, thus suggesting that some of the carotenoids were also present as protein complexes. Kahn and Chang (1965) and Kahn and Purcell (1965) also reported a small amount of carotenoid-protein complex which could be extracted, together with their chlorophyll-protein complex, by aqueous solutions of Triton X-100.

Apart from the lipid-soluble pigments, there are large amounts of other lipid materials associated with the chloroplast. It has often been assumed, in fact, on the basis of early work, that almost all the leaf lipids are concentrated in the chloroplast (Rabinowitch, 1945, page 375). According to Zill and Harmon (1962), however, up to one third of the total leaf lipids may not be present in the chloroplast. The major fraction of extra-chloroplastic lipid is to be found in the waxes, hydrocarbons and fatty

alcohols of the leaf cuticle. Also, a fairly large amount of phospholipid is presumably present, probably together with some sterol, in the membranes of the endoplasmic reticulum and other cellular organelles apart from the chloroplast.

The cuticular or surface lipids have been subjected to TLC by Purdy and Truter (1961, 1963) and their taxonomic significance has been discussed (Purdy and Truter, 1961). According to Purdy and Truter (1963), in Brassica oleracea 33% of the surface lipid is the hydrocarbon nonacosane, 26% nonacosane derivatives (ketones, secondary alcohols and a small amount of ketols), 13% esters of straight chain acids and primary alcohols, 9% free acids and 9% free straight chain alcohols; traces of other hydrocarbons (mainly hentriacontane) and some polyhydroxy benzenoid compounds were also present. Kolattukudy (1965) reviews the occurrence and separation of these surface lipids together with their probable biosynthesis.

Since the total amount of the surface lipids will vary a great deal with the various types and ages of leaf, it is obvious that whole leaf-lipid extracts can not be equated directly with the chloroplast lipid composition unless step are taken to remove such surface lipids. This could be done either by washing the leaves with an organic solvent or, since the surface lipids are mainly waxes (hydrocarbons and ester waxes) by precipitating the waxes from

an acetone solution in which they are insoluble (see the Experimental Section).

When the lipids of isolated chloroplasts have been examined, rather than the lipids of whole leaves, these lipids have often been expressed as a percentage of the dry weight of the chloroplast. As Kirk and Tilney-Basset (1967) point out however, chloroplasts isolated in an aqueous method normally lose most (or all) of their soluble protein, thus decreasing their dry weight. Kirk and Tilney-Basset, by applying their correction of 1/1.61 to earlier estimates, suggest the approximate lipid content to be 21% of the dry weight of the chloroplast. Leech (1966) suggests that much of the chloroplast lipid may be lost with the stroma protein, and from her results it appears that around 72% of the chloroplast dry weight may be lipid. The results of Leech (1966) for the protein content of Vicia faba chloroplasts (taken from the results of Bucke, Leech, Hallaway and Morton, 1966) are abnormally low however, when compared with other values, and impossible if one considers the values given by Lichtenthaler and Park (1963), for the chloroplast lamellae, to be universally applicable. In the latter preparations the protein concentration is approximately 4.6mg/mg chlorophyll, whereas Leech's best preparations (presumably containing a large amount of stroma protein) only contain 3.66mg protein/mg chlorophyll. No information is available to determine if the variation in

the protein/chlorophyll ratio of the lamellae between different species is sufficient to explain these results.

A possible alternative explanation could be that the results given by Bucke, Leech, Hallaway and Morton (1966) as total protein are actually total protein nitrogen; their results for "stripped" chloroplasts would then be comparable to those of Thornber, Gregory, Smith and Bailey (1967) for lamellae from Beta vulgaris.

Still and Price (1967) have recently reported the use of rate-zonal centrifugation for the separation of intact chloroplasts from Spinach (Spinacia oleracea), for which they give protein contents of 113mg/mg of chlorophyll. These workers also point out that they could not obtain values as high as this using the isopycnic density gradient centrifugation method of Leech (1964). Still and Price also note that the yields of intact chloroplasts varied greatly with the freshness of the spinach used (between 0 and 50% of the total chlorophyll could be obtained in the intact chloroplast fraction).

The work quoted above serves to reinforce the request of Spencer and Unt (1965) for the monitoring of chloroplast preparations by phase-contrast microscopy, which can rapidly distinguish between whole chloroplasts which have retained their bounding membrane and stripped or broken chloroplasts which have lost their stroma. According to Leech (1966), differential centrifugation in aqueous solutions cannot produce chloroplast preparations free of contamination by

other cell organelles, thus, at present, the best chloroplast preparations (i.e. those in which their condition is as close as possible to that in vivo) seem to be those of Leech (1964, 1966), Spencer and Unt (1964) and Still and Price (1967); the work of Honda, Hongladrom and Laties (1967) on plant cell organelles is also of interest in this respect. All these methods yield chloroplasts with intact boundary membranes, but the results of Still and Price suggest that protein may still be lost from such chloroplasts. The non-aqueous methods of chloroplast isolation used by Heber and co-workers (Heber, 1957; Heber, 1960; Heber and Tyszkiewicz, 1962; Heber, Pon and Heber, 1963), Stocking (1959) and Mercer and Treharne (1966) would not be suitable for lipid studies, as some loss of chloroplast lipids would occur in such systems. As an alternative to whole chloroplasts, the chloroplast lamellar system alone may be examined as this can be obtained in a fairly reproducible state (Park andPon, 1961, 1963; Bailey, Thornber and Whyborn, 1966; Thornber, Gregory, Smith and Bailey, 1967).

The lipids of both chloroplasts and whole leaves have been examined and compared by Zill and Harmon (1962) using chromatography on silicic acid, and by Booth (1962, 1965) using two-dimensional paper chromatography. Several workers have examined whole leaf lipids, but the majority have been mainly concerned with only the polar lipids; Kates (1959), Nichols (1963, 1964), Beiss (1964), Lepage (1964), Allen,

Good, Davis and Fowler (1964), and Allen, Hirayama and Good (1966). The lipids of both whole leaves and of the chlorophyll lipoprotein of Takashima (Takashima, 1952; Chiba, 1955) have been examined by silicic acid chromatography, paper chromatography and thin layer chromatography (TLC) by Hirayama (1965) and Noda, Katsura and Tanaka (1965), although it is uncertain whether the lipids of chlorophyll lipoprotein can be equated with any specific chloroplast lipid composition. This is doubtful if one considers the lowered proportion of carotenoids found in chlorophyll-lipoprotein by Noda et al, since Lichtenthaler and Calvin have shown the carotenoids to be associated almost solely with the lamellae. Thirkell and Tristram (1963) have examined the lipids co-precipitated with protein from an aqueous suspension of leaf material.

Benson (1963a) and James and Nichols (1966), have reviewed the polar lipids of photosynthetic systems, and the latter point out that four acyl lipids are specifically found, although no general pattern of fatty acid composition is discernable in this group. Benson (1963a) has indicated the importance of surfactant compounds such as glycolipids and phospholipids in the chloroplast. Green and Tzagoloff (1966) have reviewed the role of lipids in the structure and function of biological membranes in general.

The lipid composition of the chloroplast is summarized by Kirk and Tilney-Basset (1967), but since this is modelled

on the values of Lichtenthaler and Park (1963) and Park and Biggins (1964) for quantasomes, it may apply more strictly to the lipid composition of the lamellae alone. Leech (1966) has pointed out that stripped chloroplasts (equivalent to the lamellae alone) may have lost up to 75% of their total lipids along with their stroma and boundary membranes. The major site of this lipid is presumably the osmiophilic globules of the chloroplast, observed by Leyon (1954) in osmium fixed chloroplasts. Park and Pon (1961) reported the presence of such globules in a fraction presumed to be lipid obtained after sonication of spinach chloroplasts and Murakami and Takamiya (1962) have reported carotenoid absorption bands in similar lipid fractions. Bailey and Whyborn (1963) and Greenwood, Leech and Williams (1963) have examined the composition of osmiophilic globules isolated from Vicia faba (Broad Bean) and Beta vulgaris L. Cicla (Spinach Beet) respectively. Bailey and Whyborn (1963) and Bailey, Thornber and Whyborn (1966) report that the major globule fraction has a lipid content of 89% of its dry weight and may be equivalent to 2-3% of the dry weight of the lamellar system.

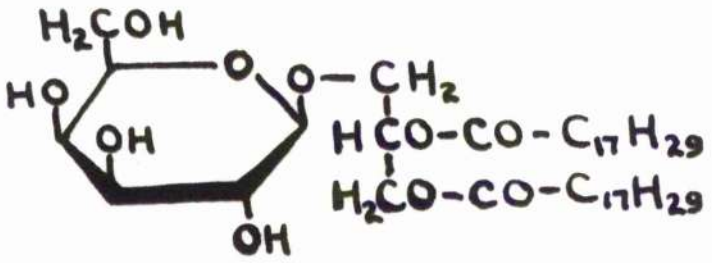
If we do assume that the lamellar system represents approximately 50% of the whole chloroplast and that the lipid content is around 20% of the chloroplast dry weight, then the globules contain about 70% of the total chloroplast lipid; this estimate may be too low and will, of course,

vary with the age and type of leaf. Thus the lipids of the chloroplast are distributed between the lamellae, the osmiophilic globules and possibly some other site in the stroma which may correspond to the "heavy globules" reported by Bailey and Whyborn (1963), Bailey, Thornber and Whyborn (1966) and Greenwood, Leech and Williams (1963).

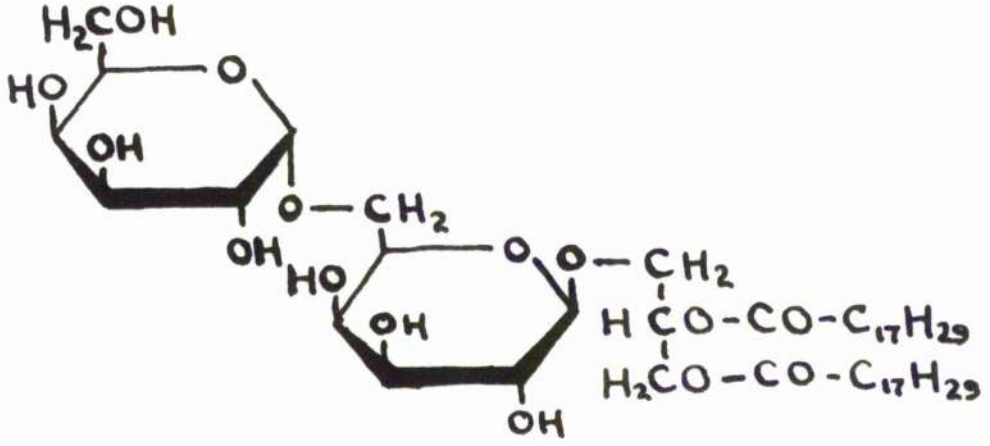
Kirk and Tilney-Bassett (1967, page 11) suggest, on thebasis of various workers' results, that the chloroplast lipid consists of 44.3% glycolipid, 9.1% phospholipid, 20.8% chlorophyll, 2.8% carotenoids, 2.2% sterol and 3.8% other terpenoid compounds with 17% of the lipid unidentified (presumably includes free fatty acids and chlorophyll and carotenoid precursors and breakdown products). Quantitatively, the most important lipids are the two galactosyl diglycerides discovered in chloroplasts by Benson, Wiser, Ferrari and Miller (1958). Benson, Wintermans and Wiser (1959) determined the structure of these two galactosyl diglycerides, and their structures are shown in fig. 4. Ferrari and Benson (1961) have demonstrated a rapid labelling of galactose in these lipids during photosynthesis in ^{14}C labelled carbon dioxide and this has led Benson (1963b) to suggest that these lipids may play a part in hexose transport in the chloroplast. A triglactosyl diglyceride has also been reported by Allen, Hirayama and Good1966.Benson and co-workers have also isolated another glycolipid from chloroplasts which they have identified as sulphoquinovosyl-diglyceride (Benson, Daniel and Wiser, 1959 and Daniel, Miyano,

Fig. 4

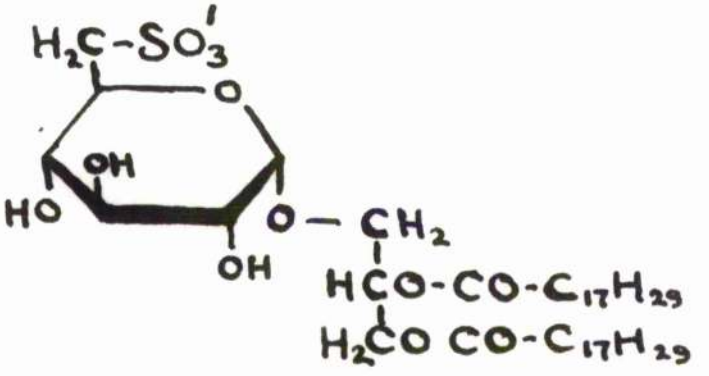
CHLOROPLAST GLYCOLIPIDS



Monogalactosyl diglyceride



Digalactosyl diglyceride



Sulphoquinovosyl diglyceride (Sulpholipid)

Mumma, Yagi, Lepage, Shibuya and Benson, 1961). The structure of this plant sulpholipid is also shown in fig. 4; it appears to constitute about 8% of the total glycolipid in spinach chloroplasts, with the monogalactosyl and digalactosyl diglycerides comprising 65% and 27% respectively (Wintermans, 1960). According to Wintermans (1960), the phospholipids of spinach chloroplasts consist mainly of phosphatidyl glycerol and phosphatidyl choline, with smaller amounts of phosphatidyl inositol, phosphatidyl ethanolamine and free phosphatidic acid (only about 2% of the total phospholipids and possibly an artefact).

The fatty acids of the polar lipids have been studied by many workers, either by examining the fatty acid content of whole leaves or chloroplasts (see reviews by Wolf, Coniglio and Davis, 1962, and Wolf, Coniglio and Bridges, 1966), or by separating the polar lipids of such tissues and examining the fatty acid composition of the individual lipids or lipid groups. The latter approach has been utilised by Nichols (1965), Nichols, Harris and James (1965), and Allen, Hirayama and Good (1966). Klopfenstein and Shigley (1967) have studied the variations in fatty acid composition of the phospholipids and sulpholipid of alfalfa during the growing season; the most noticeable change was the drop in the linolenic acid content in both fractions.

The occurrence of sterol in plant lipid extracts is well known, and Menke and Jacob (1942) reported that the

lipids of the spinach chloroplast contained 1.82-2.53% as sterol. Zill and Harmon (1962) also found sterols in spinach chloroplasts, but Nichols (1963) was unable to find any sterol in the lettuce chloroplasts he examined. The recent work of Mercer and Treharne (1966) confirms the presence of sterols in the chloroplast even after hexane washing, and these workers found that this bound sterol was quantitatively different to the phytosterols considered typical of higher plants: 80% of this sterol consisted of a component which appears to be cholesterol. Sterol esters have also been reported in the leaves of higher plants (Bailey and Whyborn, 1963; Thirkell and Tristram, 1963; Hirayama, 1965; Noda, Katsura and Tanaka, 1965).

The terpenoid compounds in leaves, apart from the phytol of chlorophyll, the carotenoids and their precursors and the sterols, are mainly made up of the isoprenoid quinones and the tocopherols. Other minor terpenoid compounds are also found and these include the monoterpenes responsible for the taste and odour of many plants, and the sesquiterpene growth inhibitor dormin. The gibberellins, a large group of plant hormones, are also derived from diterpenes. For a review of the biological significance of plant terpenoids, see Goodwin, (1967b).

Another class of terpenoids that has recently been found and shown to be associated with the chloroplasts, is that of the long chain polyisoprenoid alcohols or "prenols".

The first of these compounds to be found in plants was the all-trans-nonaprenol isolated by Rowland, Latimer and Giles (1956) from tobacco leaves and which they called solanesol. Wellburn and Hemming (1966a, 1966b) reported the isolation of a group of polyisoprenoid alcohols with both cis and trans double bonds for which they suggested the name castaprenols (they were isolated from leaves of Aesculus hippocastanum, the horse chestnut). A similar group of compounds, the ficaprenols, has also been isolated from Ficus elasticus, the decorative rubber plant (Hemming, 1967; Stone, Wellburn, Hemming and Pennock, 1967). Lindgren (1965) and Wellburn and Hemming (1966c) have also isolated a similar series of alcohols, the betulaprenols, from the wood and leaves of Betula verrucosa, the silver birch. Hemming (1967) reviews the work on the prenols of plant leaves and describes the separation and NMR spectroscopy of these compounds. There is the possibility that the equivalent castaprenols, ficaprenols and at least some of the betulaprenols are actually the same compounds.

The first of the isoprenoid quinones to be isolated from plant leaves was Vitamin K, or phylloquinone, which was isolated from alfalfa by Binkley, Cheney, Holcomb, McKee, Thayer, MacCorquodale and Doisy (1939) and Dam, Geiger, Glavind, Karrer, Karrer, Rothschild and Salomen (1939). MacCorquodale, Cheney, Binkley, Holcomb, McKee, Thayer and Doisy (1939) showed vitamin K, to be 2-methyl-3-methyl-1,4-

naphthoquinone and this structure is shown in fig. 5. In accordance with the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature (1965) vitamin K, is referred to as phylloquinone in this discussion and the work that follows. Phylloquinone has also been isolated from green leaves by Booth (1962, 1965) and from spinach chloroplasts by Kegel and Crane (1962), although Bishop (1959, 1961) and Zill and Harmon (1962) were unable to find any appreciable amounts in the chloroplasts that they examined. This may have been due to the fact that phylloquinone is more difficult to extract from the chloroplast than most of the other lipids present (Kegel and Crane, 1962).

Dam and Glavind (1938) found that phylloquinone (detected by a biological assay method) could be found in both green and non green areas of the plant, such as etiolated leaves and cauliflower head tissue. Egger (1965) has used column chromatography and thin layer chromatography to determine the phylloquinone and plastoquinone (q.v.) content of many green leaves, and has also reported its presence in the brown, red and blue-green algae, as well as in various flower petals and fruits. Egger found that the molar ratio of chlorophyll to phylloquinone varied from 100:0.5 to 100:3 as compared with 100:1 (Kegel and Crane, 1962) and from 100:1.7 to 100:5 (Lichtenthaler, 1962; Lichtenthaler and Calvin, 1964). Cole, Tendille and Gervais (1965) found that the level of phylloquinone in tobacco leaves was

decreased in nitrogen deficiency and dropped slightly with age in normal leaves. Hindberg and Dam (1965) found that phylloquinone levels in oak leaves remained fairly constant between June and September.

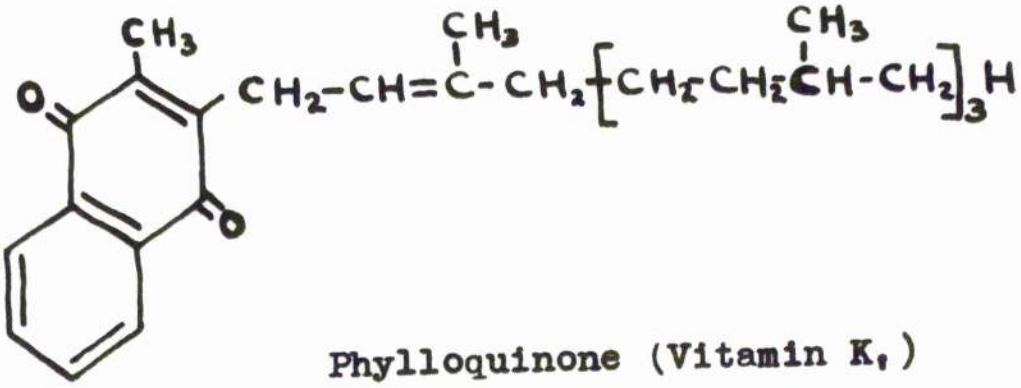
As Pennock (1966) points out, data on the distribution of phylloquinone are scarce, and the methods used have not always been very reliable. Apart from the difficulty of extracting phylloquinone (Kegel and Crane, 1962) the amounts present are very small and it is very difficult to separate from plastoquinone A (q.v.) even on thin layer chromatograms. An added difficulty is that the borohydride reduction assay commonly used for the estimation of the other isoprenoid quinones cannot be used reliably for phylloquinone due to the alakli lability of such naphthoquinones. Lester, White and Smith (1964) however, have modified the borohydride reduction assay so that it may now be used for naphthoquinones.

McKenna, Henninger and Crane (1964) and Crane, Henninger, Wood and Barr (1966) have reported the presence of low concentrations of a second naphthoquinone in spinach chloroplasts which they suggested may be a demethylated compound similar to that isolated by Baum and Dolin (1965) from Streptococcus faecalis.

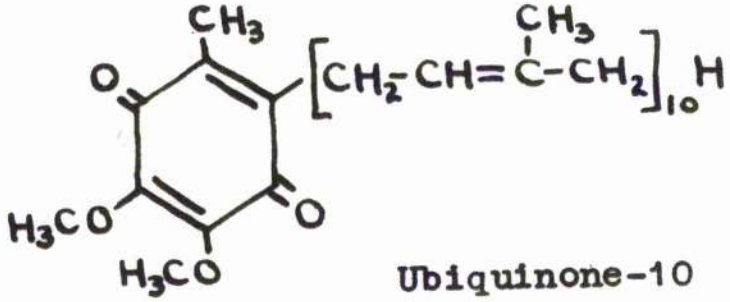
In 1955, Morton's team in Liverpool reported the isolation of a substance "SA" from various animal tissue, which was thought to be a steroid ene-dione (Festenstein, Heaton, Lowe and Morton, 1955). SA was also isolated from baker's yeast (Heaton, Lowe and Morton, 1956), and when it

was later found to be a quinone, it was given the name ubiquinone from its ubiquitous distribution. Independently of the work at Liverpool, a team working under Green at Wisconsin isolated a water-insoluble quinone from beef-heart mitochondria, which they designated as Q-275, from its absorption maximum at 275nm in ethanol (Crane, Hatefi, Lester and Widmer, 1957). Q-275 was renamed Coenzyme Q and was shown to be identical with ubiquinone by the independent determination of its structure by Morton, Gloor, Schindler, Wilson, Chopard-dit-Jean, Hemming, Isler, Pennock, Rüegg. Schweiter and Wiss (1958), Wolf, Hoffman, Trenner, Arison, Shunk, Linn, McPherson and Folkers (1958) and Shunk, Linn, Wong, Wittreich, Robinson and Folkers, (1958). In accordance with the IUPAC-IUB Commission's recommendation, the name ubiquinone is used throughout this work and, since a series of isoprenologues have been shown to occur in nature (Lester, Crane and Hatefi, 1958), it is followed where necessary, by a numeral defining the number of isoprene units in the side chain. The structure of ubiquinone-10, the isoprenologue isolated from beef-heart mitochondria, is shown in fig. 5.

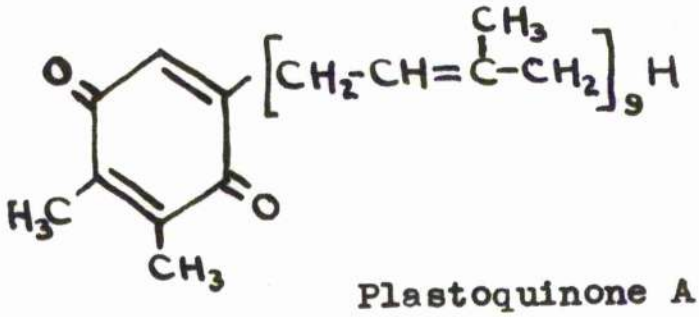
Crane and Lester (1958) found that ubiquinone was present in plant leaves, together with another quinone which was associated solely with the chloroplasts (Crane, 1959a) and for which they suggested the name Q-254. This name was changed to plastoquinone (Crane, 1959b) to emphasise its localisation in chloroplasts as well as the fact that



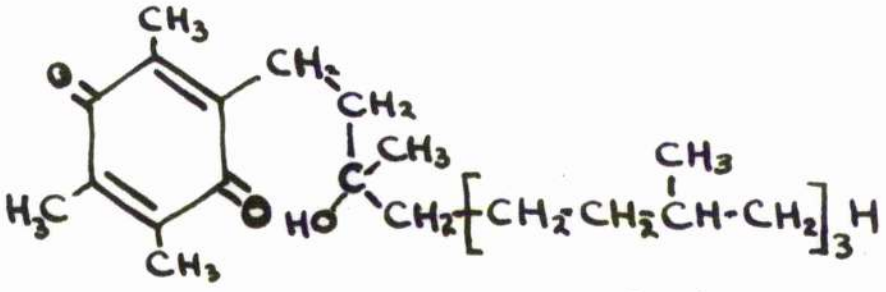
Phylloquinone (Vitamin K_1)



Ubiquinone-10



Plastoquinone A



α-tocopherolquinone

it is analogous rather than homologous with the ubiquinones. The structure of plastoquinone was determined by Trenner, Arison, Erickson, Shunk, Wolf and Folkers (1959) and by Kofler, Langemann, Rüegg, Chopard-dit-Jean, Rayroud and Isler (1959) as a dimethyl benzoquinone with an isoprenoid side chain containing nine five-carbon units: this structure is shown in fig. 5. Plastoquinone appears to be identical to a quinone which had previously been isolated from alfalfa by Kofler (1946) but on which no further work seemed to have been done.

Further work under Crane, at Purdue University, Indiana, has shown the presence of two other plastoquinones in plants, designated as plastoquinone B and plastoquinone C, the original being termed plastoquinone A (Kegel, Henninger and Crane, 1962). The site of these quinones in the plant cell has been shown to be the chloroplasts by Kegel, Henninger and Crane (1962), Stevenson, Hemming and Morton (1963) and also by Khau van Kien (1966) using histochemical techniques. Later it has been reported that plastoquinone C actually contains plastoquinones C and D, both of which are extremely labile (Kegel and Henninger, 1963 ; Henninger and Crane, 1963, 1964). The same team has also reported the presence of α , β and γ -tocopherolquinones in spinach chloroplasts (Dilley and Crane, 1963a; Henninger, Dilley and Crane, 1963). Lichtenthaler and Calvin (1964) have confirmed the presence of plastoquinones A and B, and α-tocopherolquinone in

spinach chloroplasts, but report that they can find no evidence for the presence of plastoquinone C. It appears however, that the compound designated as plastoquinone B by these workers, was probably a mixture of plastoquinones C and D (Threlfall, Griffiths and Goodwin, 1965).

Threlfall, Griffiths and Goodwin (1965) report the isolation of two analogues of plastoquinone, which they designate plastoquinones C' and D', since their spectral properties differ slightly from those of the plastoquinones C and D reported by Henninger and Crane (1964). It seems likely that the preparations of Henninger and Crane (1964) were slightly impure (see Crane, Henninger, Wood and Barr, 1966) and that they were in fact the same compounds as those isolated by Threlfall, Griffiths and Goodwin (1965). Eck and Trebst (1963) have reported the isolation of plastoquinone-4 from horse chestnut chloroplasts and Crane, Henninger, Wood and Barr (1966) and Barr, Huang and Crane (1966) confirm this finding. Eck and Trebst (1963) also report the presence of dimers of both plastoquinone-4 and plastoquinone-9 in horse chestnut, but it seems likely that these compounds are artefacts. Misiti, Moore and Folkers (1965) report the isolation of plastoquinone-3 from spinach chloroplasts, but Crane, Henninger, Wood and Barr (1966) state that they are unable to find this compound in their preparations.

Henninger, Barr, Wood and Crane (1965) reported the presence of a fifth plastoquinone in chloroplasts which

they called plastoquinone E, but a re-examination of this compound showed it to be identical to plastoquinone B (Henninger, Barr and Crane, 1966).

Griffiths, Wallwork and Pennock (1966) have reported that the fraction designated plastoquinone B can be split into six different compounds by a combination of TLC and reversed phase TLC. These workers similarly report that the plastoquinone C + D fraction can also be resolved into six components. Plastoquinone C was shown to be a derivative of plastoquinone A with an allylic hydroxyl group in the side chain by Das, Lounasama, Tendille and Lederer (1965) using mass spectrometry. These workers also suggested that plastoquinone B was plastoquinone A with an additional unsaturation in the side chain, but in later work (Das, Lounasama, Tendille and Lederer, 1967) they showed that it was actually an ester of plastoquinone C or D with palmitic acid. The six plastoquinone B and six plastoquinone C + D compounds are thought to be due to the position of the hydroxyl or ester grouping, although it is probable that if there was an unsaturation in the fatty acid moiety of plastoquinone B this would alter its polarity.

The presence of α-tocopherolquinone in chloroplasts has been confirmed by Bucke, Hallaway and Morton (1964), Bucke, Leech, Hallaway and Morton (1965, 1966), Bucke and Hallaway (1966) and Cole, Tendille and Gervais (1965). Bucke and Hallaway (1966) have also studied the variation in

α-tocopherolquinone and plastoquinone C concentrations in Vicia faba throughout the growing season. They find that the ratio of α-tocopherolquinone to chlorophyll does not vary greatly throughout the year, but that plastoquinone C is at a maximum in early summer and is low, or not detectable, in winter. Crane, Henninger, Wood and Barr (1966) suggest that all the quinones in spinach chloroplasts tend to increase together during the season so that their relative amounts remain the same. Hindberg and Dam (1965) report that plastoquinone A increases significantly during the summer and autumn, whilst ubiquinone-10 remains relatively constant, as does phylloquinone.

Lichtenthaler and Calvin (1964) and Becker, Gross and Shefner (1962) reported the presence of the isoprenoid quinones (apart from ubiquinone) in the chloroplast lamellae, whilst Bailey and Whyborn (1963) and Greenwood, Leech and Williams (1963) report the additional presence of plastoquinones in osmiophilic globules. Bailey and Whyborn (1963) and Bailey (1965) could find no evidence for phylloquinone in the globules and α-tocopherolquinone was not observed, although it may have been masked by the polar plastoquinones C and D. Barr, Huang and Crane (1966) suggest that the seasonal increase in plastoquinone A in horse chestnut chloroplasts may be partly attributed to the increase, in number and size, of the osmiophilic globules present, and the increase in plastoquinone with ageing, observed by

Egger (1965) would probably correlate with the appearance of globules.

The separation methods used for the isoprenoid quinones have normally included an initial column chromatographic step and various adsorbents have been utilised. Crane and co-workers have frequently reported the use of Decalso (a sodium alumino-silicate) e.g. Crane (1959a, 1959b, 1961, 1964), Crane and Lester (1962), Henninger and Crane (1964). Misiti, Moore and Folkers (1965) have also described the use of Decalso for the isolation of plastoquinone-3. Henninger, Barr and Crane (1966) have reported the destruction of plastoquinone B on Decalso columns and have used a mixture of Hyflo and silicic acid. A somewhat similar adsorbent (silicic acid/Chromosorb W) has been used by Tendille and Gervais (1963) and Tendille, Gervais and Cofc (1964); Bats, Gervais and Cofc (1962) described a fractional elution procedure using the same adsorbent mixture. Silicic acid alone has been used by Crane (1959b), Egger (1965) and by Allen, Franke and Hirayama (1967). Rebel and Mandel (1965) and Lester, White and Smith (1964) have also used silicic acid columns for the isolation of bacterial quinones.

Alumina, deactivated with either water or dilute acids, has been used by Kofler (1946), Kofler, Langeman, Rüegg, Chopard-dit-Jean, Rayroud and Isler (1959), Hindberg and Dam (1965), Lichtenthaler and Calvin (1964), and acid washed alumina has been used by the Liverpool and Aberystwyth

groups; e.g. Threlfall, Griffiths and Goodwin (1965), Griffiths, Wallwork and Pennock (1966), Griffiths, Threlfall and Goodwin (1967), Threlfall and Goodwin (1967), Bucke, Leech, Hallaway and Morton (1965, 1966), Carr and Hallaway (1965, 1966).

Links (1960) has shown that chromatography on alumina can lead to the alteration of isoprenoid quinones (he found that ubiquinone was converted to its cyclic isomer ubichromenol) and Scott (1965) has shown that plastoquinone-1 is rapidly destroyed on alumina. Hemming, Morton and Pennock (1961) have examined the alteration of naturally occurring quinones on alumina and conclude that little degradation occurs if only short periods (less than about four hours) are used with the deactivated alumina (Brockmann Grade III).

Draper and Csallany (1960) suggested that ubiquinone may be converted to ubichromenol by an involved saponification step, and Folkers and co-workers (Linn, Trenner, Arison, Weston, Shunk and Folkers, 1960; Folkers, Shunk, Linn, Trenner, Wolf, Hoffman, Page and Koniuszy, 1961) have shown that the methoxy groups of ubiquinone can be replaced by ethoxy groups during ethanolic saponification. These facts, together with the known structure of plastoquinone B suggest that the saponification step, often used for the removal of the "saponifiable lipids", should be abandoned in any work on the isoprenoid quinones.

Thin layer chromatography (TLC) has regularly been

used to purify the biologically occurring quinones and Dilley (1964) and Tendille, Bats, Gervais and Gaborit (1965) devote complete publications to the TLC of these compounds. Sommer and Kofler (1966) in their review of the methods of analysis of the quinones, present a table summarizing some of the results obtained by TLC. Silica Gel is the normal adsorbent used, but some reversed phase and partition TLC techniques have been reported utilising paraffin impregnation, (Wagner, Hörhammer and Dengler, 1962; Bollinger, 1962) or polyamide and cellulose layers (Egger and Kleinig, 1965).

Reversed phase paper chromatography of the isoprenoid quinones using impregnation with silicone oil has been reported by Lester and Ramasarma (1959), and Linn, Page, Wong, Gale, Shunk and Folkers (1959) have used papers impregnated with petroleum jelly. Lichtenthaler and Calvin (1964) and Lichtenthaler (1964) have described the use of alumina impregnated papers for the separation of plant quinones, and Booth (1962, 1965) has achieved separations of these quinones by his two dimensional paper chromatographic technique.

Partition columns have rarely been used for the separation of biological quinones, but Matschiner and Taggart (1967) have recently reported the separation of phylloquinone and associated lipids on a mixture of Celite and polyethylene powder. Polyethylene powder columns had previously been used for the purification of quinones e.g. by Wiss and Gloor (1958) and Gloor and Wiss (1960)

The other major class of isoprenoid compounds present in plant leaves is the tocopherols. Although α- and β-tocopherols were first isolated in 1936, (Evans, Emerson and Emerson, 1936) and their structures established only very shortly afterwards by Fernholz (1937, 1938), nevertheless, analysis of plant tissues for the different tocopherols present was not successfully achieved until the work of Green (1958). Even now, the systems of tocopherol analysis are neither very simple nor very reliable, although Booth (1963) has used his two dimensional paper chromatographic technique with some success to separate α-, γ- and δ-tocopherols. Also, Dilley and Crane (1963a, 1963b) have reported the association of tocopherols in the plant cell mainly with the chloroplast, and their separation by TLC and silicone, paraffin oil or petroleum jelly impregnated paper.

One of the difficulties of tocopherol analysis is the presence of the lipid-soluble quinones described above, which may react with the rather non-specific tocopherol assay reagents to give erroneous results. As Booth (1961) points out, many solvents and some of the adsorbents sued for the separation of the tocopherols can give rise to spurious results with the normal tocopherol assay procedures. The methods of Dilley and Crane (1963b) and Roughan (1967b) involving TLC followed by oxidation of the tocopherol to the tocopherolquinone do not appear to be subject to the

same difficulties.

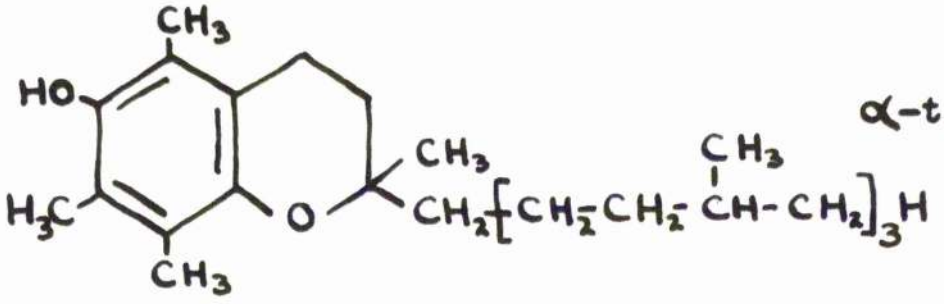
The separation of the tocopherols by TLC has also been described by Seher (1961), Dâvidek and Blattnâ (1962), Stowe (1963), Pennock, Hemming and Kerr (1964) and Roughan (1967a). Skinner, Parkhurst and Alaupovic (1964) have used TLC to separate α-tocopherol from some of its oxidation products.

The four common tocopherols, α, β, γ and δ are shown in fig. 6. Pennock, Hemming and Kerr (1964) have shown that the compounds formerly referred to as ζ and ϵ -tocopherols are not actually true tocopherols, but contain an unsaturated bond in each of the three isoprenoid units of the side chain. Pennock et al propose the name tocotrienols for these compounds and describe the presence of α, β and γ-tocotrienols (related to α, β and γ-tocopherols) in nature. δ-tocotrienol, the analogue of δ-tocopherol was later isolated from Hevea latex by Dunphy, Whittle, Pennock and Morton (1965) and Whittle, Dunphy and Pennock (1966). The same team has also described the isolation of a cyclic derivative of plastoquinone-9 from the leaves of Hevea brasiliensis (Rubber plant) and have called this compound plastochromanol-8 (Whittle, Dunphy and Pennock, 1965; Dunphy, Whittle and Pennock, 1966). The structure of plastochromanol-8 is also shown in fig. 6.

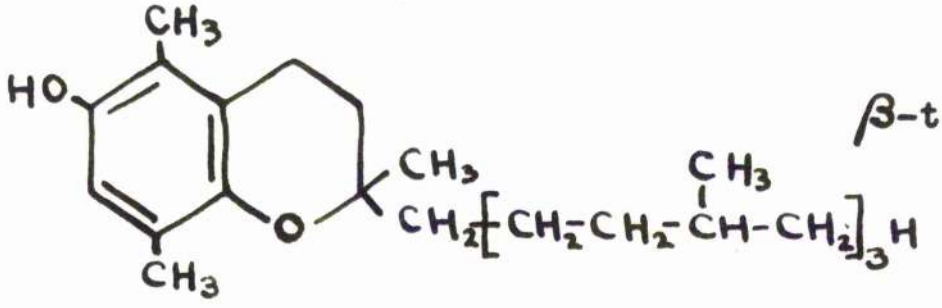
No role has yet been ascribed to the chromanols found in plant leaves, but the plastoquinones have been shown to

Fig. 6

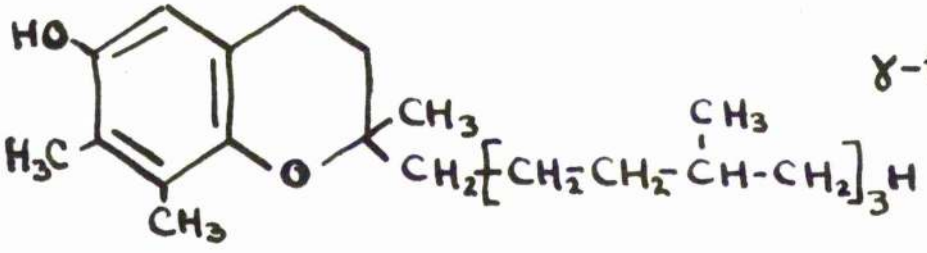
PLANT CHROMANOLS



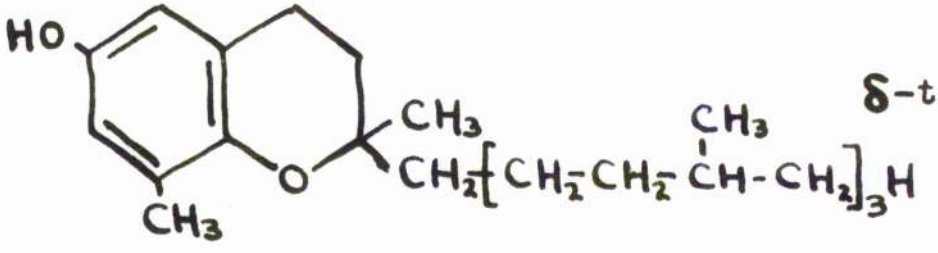
α-tocopherol



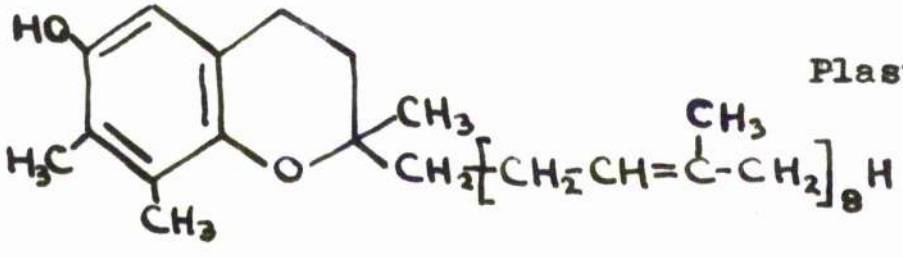
β-tocopherol



γ-tocopherol



δ-tocopherol



Plastochromanol-8

function in photosynthesis. Bishop (1959) showed that extraction of chloroplasts with heptane caused the loss of Hill reaction activity, but this activity was restored by adding plastoquinone A and Crane, Ehrlich and Kegel (1960) and Crane (1961) reported that endogenous plastoquinone was reduced to plastoquinol when chloroplasts were illuminated. Various workers have examined these effects and confirmed the initial observations. Addition of plastoquinone A to extracted chloroplasts has been shown to result in the restoration of at least some of the lost Hill reaction activity, usually measured by ferricyanide reduction (e.g. Bishop, 1961; Redfearn and Friend, 1962b; Trebst, 1963). Several workers have also been able to show that the requirement is relatively non-specific and that plastoquinone A can be replaced by several other quinones, both naturally occurring and synthetic (Trebst and Eck, 1961; Krogmann and Olivero, 1962; Redfearn and Friend, 1962b; Henninger and Crane, 1963; Trebst, 1963; Cho, Parks and Zweig, 1966). Other workers (Arnon, Whatley and Horton, 1962; Arnon and Horton, 1963) used a more exhaustive extraction procedure and showed that NADP reduction was also abolished but could be restored by the addition of plastoquinone. This reactivation, in contrast to the Hill reaction, was specific to plastoquinone. This work led to the idea that plastoquinone (presumably plastoquinone A) had two sites of action in photosynthesis. Klingenberg, Muller, Schmidt-Mende and

Witt (1962); Witt, Muller and Rumberg (1963) and Muller, Rumberg and Witt (1963) have examined the changes in absorption spectra due to plastoquinone in photosynthetic systems and they also postulate the existance of two possible sites for plastoquinone. Redfearn (1965) reviews this and other early work and postulates the additional action of plastoquinone in cyclic and non-cyclic photophosphorylation, although in cyclic photophosphorylation it may substitute for some other quinone such as phylloquinone.

Crane and Henninger (1966), in a later review of the role of quinones in photosynthesis, conclude that at least one tenth of the plastoquinone A of chloroplasts functions in electron transport and it may function as the initial electron acceptor from chlorophyll in System II of photosynthesis. There is also evidence for a second functional site for plastoquinone and the large pool of plastoquinone available to this site is primarily in the reduced state in phosphorylating chloroplasts, but becomes oxidized in uncoupled systems. Crane and Henninger (1966) also present evidence for the function of plastoquinone C and suggest that indications have been obtained of a photosynthetic role for phylloquinone, α-tocopherolquinone and possibly other plastoquinones. Henninger and Crane (1967a, 1967b) report further evidence for the role of plastoquinone C and suggest that it functions after plastoquinone A as an electron carrier from System II to System I.

The biosynthesis of the terpenoid quinones in plant leaves has been studied almost exclusively by Goodwin's group at Aberystwyth. The results of this study have only appeared in the last couple of years and are still incomplete, but Threlfall (1967) presents a review of the situation up to April, 1966. In early work on the biosynthesis of terpenoids in illuminated excised maize seedlings, it was found that labelled mevalonic acid (MVA), was only poorly incorporated into β-carotene, although a high activity was found in the phytosterols (Goodwin, 1958). Labelled carbon dioxide however, was rapidly incorporated into β-carotene, but only poorly into the sterols. Similar results have been obtained for the phytol residue of chlorophyll (Mercer and Goodwin, 1962), and also for the isoprenoid side chain of plastoquinone but not ubiquinone, both in maize seedlings (Griffiths, Threlfall and Goodwin, 1964) and in other plants (Treharne, Mercer and Goodwin 1964, 1966). This has led Goodwin and Mercer (1963) and Threlfall, Griffiths and Goodwin, (1964) to suggest that there are two sites of terpenoid biosynthesis in the cell, the side chain of plastoquinone, together with phytol and the carotenoids, being synthesized from carbon dioxide in the chloroplast, whilst the sterols and the side chain of ubiquinone are synthesized from MVA at some other cellular site. The hypothesis of two sites of terpenoid synthesis is presented fully by Goodwin (1965, 1967b) and Threlfall

(1967) and has two prerequisites; firstly, that the chloroplast membrane is impermeable to MVA, and secondly, that all the enzymes necessary for terpenoid biosynthesis occur both inside and outside the chloroplast. Rogers, Shah and Goodwin (1966, 1967) have shown the presence of MVA-kinase (the first enzyme of terpenoid biosynthesis) both inside and outside the chloroplast and, at the same time, have demonstrated the impermeability of the chloroplast membrane to MVA.

The isoprenoid side chain of ubiquinone in both animals andmicro-organisms has been shown to arise from MVA, probably via a polyisoprenoid pyrophosphate which condenses with p-hydroxy benzoic acid to yield a multiprenyl phenol (for reviews see Glover, 1965; Olson, 1966, and Rudney and Raman, 1966). The ring-methyl and O-methyl groups of ubiquinone have been shown to arise from methionine. The situation as regards phylloquinone (and menaquinones) in micro-organisms is uncertain, it may arise in a similar manner to ubiquinone or by prenylation of a naphthoquinone nucleus (see Rudney and Raman, 1966).

Threlfall, Griffiths and Goodwin (1967) have confirmed the incorporation of labelled MVA into plastoquinone, phylloquinone, ubiqunone and α-tocopherolquinone as well as α-tocopherol in maize and barley shoots, and Threlfall, Whistance and Goodwin (1967) confirmed the incorporation of labelled methionine into these compounds. Whittle, Audley

and Pennock (1967) at Liverpool have also reported the incorporation of labelled methionine into the quinones and chromanols of Hevea brasiliensis (rubber tree) latex.

The techniques used for studying the biosynthesis of the terpenoid quinones in higher plants have usually involved exposing etiolated seedlings to light, often in the presence of either labelled MVA or labelled carbon dioxide. These techniques and their results have been reported by Treharne, Mercer and Goodwin (1966) and Griffiths, Threlfall and Goodwin (1967). Threlfall and Goodwin (1967) have also reported the development of terpenoid quinones in Euglena under similar conditions. Dodge and Whittingham (1966) have reported the formation of plastoquinone (presumably A) in etiolated flax seedlings, and Gaunt and Stowe (1967) have examined the formation of β-carotene, chlorophyll, α-tocopherol, phylloquinone, ubiquinone and plastoquinone A in etiolated pea plants.

Etiolation studies have also been used by several workers to study the development of carotenoids in the leaves of higher plants (Strain, 1938b; Seybold and Egle, 1938; Blaauw-Jansen, Kamen and Thomas, 1950; Kay and Phinney, 1956; Goodwin, 1958; Goodwin and Phagpalngorm, 1960), but most of these reports do not describe the development of the individual xanthophylls present. Virgin (1966) has examined the effect of red light on carotenoid synthesis in etiolated leaves and reports that a phytochrome system is involved.

Aims of the Investigation

The major aims of this investigation were as follows:

1) To examine the separation of the leaf carotenoids by TLC with a view to achieving a rapid separation of these pigments which could be used to examine their qualitative and quantitative variation under different physiological conditions.

2) To examine the separation of the isoprenoid quinones present in leaf extracts using TLC and any other techniques available, in order to obtain a method equivalent to that required in 1) above for the carotenoids. As can be seen from the introduction, the majority of the published work on the leaf quinones did not appear until after this investigation was under way, consequently a great deal of work had to be done in order to become familiar with the handling and detection of these quinones, particularly considering their suspected instability.

3) To use any techniques developed under 2) above to study the variation in the content of the isoprenoid quinones (particularly plastoquinones B, C and D) throughout the growing season.

4) To use any techniques developed under 1) and 2) above to examine the development of the isoprenoid quinones and carotenoids on illumination of etiolated leaves, to see if this could shed some light on their possible biosynthesis.

5) Finally, to examine any of the other non-polar lipids encountered in leaf lipid extracts with a view to determining what other terpenoids may be present.

GRADIENT ELUTION CHROMATOGRAPHY

GRADIENT ELUTION CHROMATOGRAPHY

During early attempts at column chromatography of the lipid soluble quinones of plant leaves it was realised that a large degree of "tailing" of the quinones (particularly plastoquinone A) occurred when the normal type of "stepwise elution" was employed. Consequently, in order to prevent, or at least reduce, this tailing, "gradient elution" chromatography was attempted, which also allowed the development of a partially automated system of quinone analysis (see the Experimental Section). Stepwise elution tends to make automatic operation of a chromatographic system much more difficult. although various devices have been described for the automation of stepwise elution systems. Such devices are usually based on either time or volume measurements. The former type of device is exemplified by timer controlled solenoid valves or motorised stopcocks as described by Hamilton and Anderson (1959), Hamilton (1963), Lerner (1963) and Rombauts and Raftery (1965). Volume controlled devices are described by Kreisher and McClendon (1960), Teekell, Boling, Lyke and Chiriboga (1962), Anderson, Bond and Canning (1962) and Morris and Morris (1964, p155), and a number of automatic valves which operate when a solvent reservoir runs dry have also been described. In this last class are the hydrostatic float valve of Locker (1965), the light activated valves of Petrakis (1965a, 1965b) and the design of Hicks and Nalevac

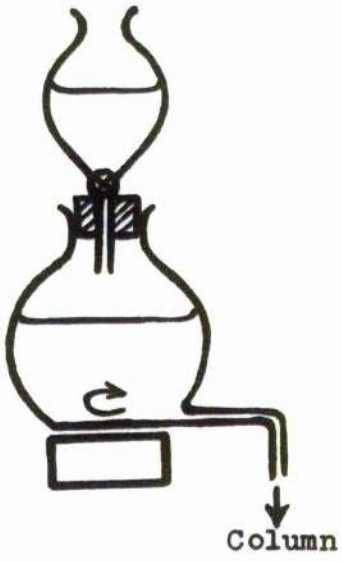
(1965) which relies on the break of an electrical connection when the reservoir runs dry. Many of these automatic stepwise elution devices are highly complex and more suitable for the routine operation of a chromatographic method than for the development of such a procedure.

The gradient elution technique has other advantages apart from the the ease of automation; the major ones are the reduction of tailing of compounds on the column, and consequent on this, an overall reduction of bandwidth and the prevention of double zoning or false peaks which can occur with stepwise elution techniques. The theoretical aspects of the effect of a concentration gradient on the desorption process are discussed by Alm, Williams and Tiselius (1952), Lakshmanan and Lieberman (1954), Drake (1955), Jermyn (1957) and Morris and Morris (1964, pp90-106). Somewhat similar theoretical considerations for gradient elution chromatography on ion exchange resins are discussed by Freiling (1955) and by Schwab, Rieman and Vaughan (1957). Snyder (1965) gives a full treatment of the general theory of gradient elution chromatography.

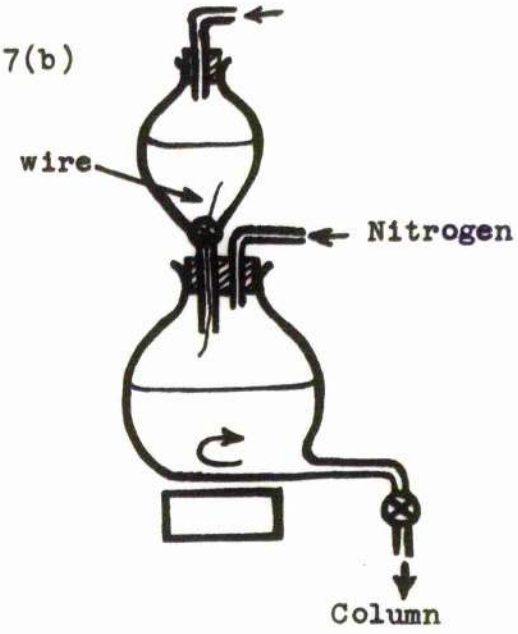
The practical technique of gradient elution chromatography was first described by Alm, Williams and Tiselius(1952) and by Donaldson, Tulane and Marshall (1952) and the type of apparatus used by both these sets of workers is shown in fig.7(a). The mixing chamber contains the weaker eluant and the reservoir contains the stronger. This is a "constant-volume" or closed chamber type of apparatus and

Fig.7

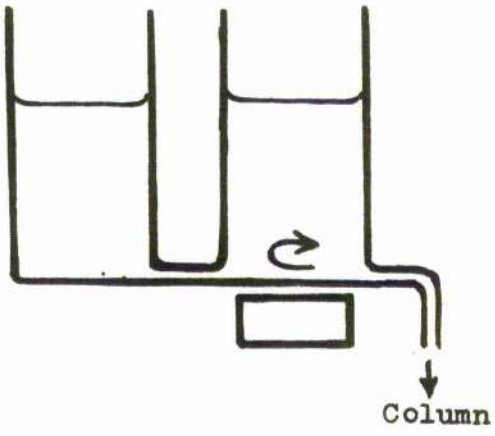
7(a)



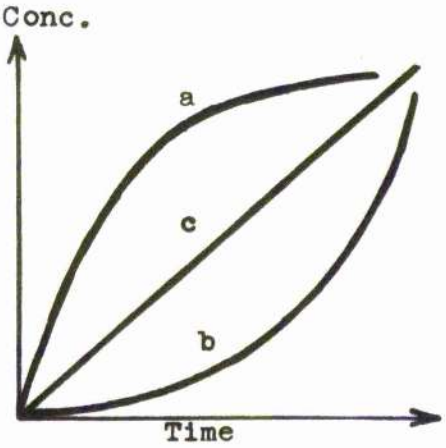
7(b)



7(c)



7(d)



produces the exponential type of gradient (a) shown in fig. 7(d). The volume of fluid in the stirred mixing chamber in such a device remains constant throughout a chromatographic run, since the flow rate of the stronger eluant into the mixing chamber is the same as the rate of flow of the mixed solvent out. Amongst the many workers who have utilised this type of apparatus are Hirsch and Ahrens (1958) in their investigations into the separation of complex lipids on silicic acid. Warner and Lands (1960) describe the construction of a nomogram for the calculation of the gradient produced by such a system, as does Kočent (1961).

Lakshmanan and Lieberman (1953, 1954) have modified the constant volume type of apparatus and have made the rates of flow into and out of the mixing chamber (R_1 and R_2 respectively) independently variable, the former by means of a tungsten wire inserted into the stopcock and the latter by means of a combination of the exit stopcock and the gas pressure applied to the whole apparatus. These workers show that three cases exist for their modified system (i) $R_2 > 2R_1$ in which case a convex or exponential-type of gradient is produced (the true exponential gradient is given by $R_2 = R_1$); (ii) $R_2 = 2R_1$ in which a straight line or linear gradient is produced (curve c in fig. 7d); and (iii) $R_2 < 2R_1$ when a concave gradient (fig. 7d, curve b) is produced. These workers also go on to show that the concave gradient is superior to both the linear and the convex or exponential type of gradient in most applications.

Another type of gradient elution device is described by Parr (1954) and by Bock and Ling (1954). This can be described as a hydrostatic or constant level device and the basic form of such an apparatus is shown in fig. 7c. Where the cross sectional areas of the two cylinders are equal (as in the design of Parr) a linear gradient is produced, provided that the two fluids are of equal density. More complex types of gradients can be produced however, by the modifications proposed by Bock and Ling; these modifications effectively alter the cross sectional area of the chambers as the level changes.

Bock and Ling (1954) point out that these devices, which operate by hydrostatic equilibrium, are affected by density differences between the two eluants and suggest that this can be minimised by allowing mixing to take place at a float on the surface of the weaker eluant. Kocent (1961) discusses the effect of density differences on the type of gradient produced by a constant level type of apparatus, and Bader and Morgan (1962) and Wren (1959 , 1963) describe "constant level" devices connected by siphons which enable gradients to be produced using organic solvents of greatly differing densities, at the same time they also describe corrections which can be applied to determine the approximate shape of the gradient to be produced. Similarly, Piez (1956) discusses another cause of variation from the expected gradient using constant level devices: the

buffering effects obtaining in pH gradients.

Modifications of the simple constant level device have been described by a number of authors. Rosett (1965) has approximated curved density gradients by the production of successive linear gradients by means of layered solutions in the reservoir chamber. Hegenhauer, Tartof and Nace (1965) have described a system of two concentric cylinders, the inner chamber serving as the mixing chamber and the space between the inner and outer chambers as the reservoir; by means of different liners, various concave, linear or convex gradients can be produced from the basic apparatus. Bessman (1967) describes the production of successive linear gradients automatically by hydrostatic means and presents equations for the calculation of the effluent concentration at any given stage.

The most versatile gradient producing device described in the literature is the multichamber hydrostatic device of Peterson and Sober (1959) known as the 'Varigrad'. This consists of several stirred chambers of equal volume (usually nine) in hydrostatic equilibrium and connected in series by stopcocks. The eluant is withdrawn from the end chamber and a fairly complex gradient can be set up by varying the initial concentrations in the different chambers. Both the original design of Peterson and Sober (1959) and the improved and simplified version described by Peterson and Rowland (1961) suffer from the disadvantages that they

can not easily be adapted to operation with organic solvents (these will attack some of the material of which the Varigrad is made) and density differences may also cause difficulties. Similar problems also affect the equivalent device described by Smith and Stahmann (1962) although that of Larrabee and Klingman (1963) would, at least, be resistant to organic solvents. Other devices for the production of relatively complex gradients have been described based on the constant volume or closed chamber type of apparatus. The earliest of these utilises a reservoir feeding two constant-volume or closed mixing chambers e.g. Drake (1955), Schwab, Rieman and Vaughan (1957), McGilvery (1960) and Kesner, Muntwyler, Griffin and Abrams (1963). Such devices are useful for the production of linear gradients from a constant volume system.

A discrepancy should be pointed out between the terminology of constant volume and constant level systems. In constant volume devices the number of mixing chambers is normally given excluding the reservoir chamber, whereas in a constant level system the chamber furthest from the column (which can be considered as the reservoir chamber) is included in the total number of chambers. Thus the above devices, if considered in constant-level terminology, are three-chambered systems. Kesner, Muntwyler, Griffin and Abrams (1962) have described a four-chambered constant-volume system and Svensson and Forchheimer (1962) describe

the construction of an apparatus with 4-6 closed chambers plus a reservoir (i.e. a 5-7 chambered system) together with the gradients produced by such a device. Tables for 2-9 chambered constant volume systems are given by Niederwieser (1967) and for a 10 chambered system (produced from a modified Varigrad) by Chase (1963).

It should be realised that the combination of any of the methods of automatic stepwise elution previously described (or indeed any methods of stepwise elution) with a single constant volume mixing chamber will lead to the production of complex concentration gradients.

In this present work, methods similar to those of Wren (1963) and Bader and Morgan (1962) were attempted in order to produce a linear or concave gradient containing an increasing concentration of 1, 2-dichloroethane (s.g. 1.25) in n-hexane (s.g. 0.66). Due to the greatly differing densities however, difficulty was found in adjusting the initial levels of the two solvents. Lakshmanan and Lieberman (1953, 1954) show that a linear gradient is produced from a hydrostatic system when the flow rate into the mixing chamber is half that leaving the chamber, thus theoretically it should be possible to produce a linear gradient by replacing the flow under hydrostatic pressure by two micro-pumps having fixed flow rates, so that the pump rate into the mixing chamber was half of the pump rate out.

Such a system can be considered as a development of

that of Lakshmanan and Lieberman (1953, 1954) and has also been used by Wallach and Nordby (1963) to produce a flexible system for gradient elution chromatography using organic solvents. Davis, Santen and Agranoff (1965) and Leif and Vinograd (1964) have used proportioning pumps in a similar way to produce linear density gradients for density gradient centrifugation. Vestergaard (1960) has used a motor driven syringe to add the stronger eluant to a mixing chamber in the gradient elution chromatography of steroids and Arcus (1960, 1967) has used a pair of connected syringes to produce linear gradients for density gradient centrifugation. Motor driven syringes have also been used by Choules (1962).

The gradient elution system described by Nelson (1963) differs from those above in that the gradient pump adding the stronger eluant changes in speed during the chromatographic run, in the others described the flow rates can be varied but remain constant throughout a particular operation. Devices in which the mixing chamber and reservoir flow rates vary in ratio during a chromatographic run have also been described by Anderson, Crisp, Riggle, Vurek, Heftmann, Johnson, Francois and Perrine (1961), and by Curtain (1962).

The system described above, utilising two chambers connected to each other by means of a constant rate pump with a further constant rate pump supplying the mixed eluant to the column, was used in initial attempts at gradient

elution chromatography of the leaf quinones. The gradients produced by this apparatus however, gave too steep an initial rise in concentration (of the stronger eluant) and so a second mixing chamber, containing the weak eluant, was placed in series with the first in order to produce a shallower rise: this system of three chambers is shown in fig. 8(a). When the three-chambered system is operating with the pump rate ratios so that $P_2=2P_1$ and $P_3=P_2+P_1$ $(=3P_1)$ then it becomes equivalent to the three-chambered system given by Peterson and Sober (1959) and Larrabee and Klingman (1963); thus it seemed possible to increase the number of pump-connected chambers in order to produce complex gradients of the type generated by Varigrad. In fact, such a system can be operated so as to simulate either the Varigrad or the closed multichamber type of system described by Svensson and Forchheimer (1962), Chase (1963) and Niederwieser (1967).

At this stage it seemed desirable to be able to calculate theoretically, the gradients for a given set of conditions: Wallach and Nordby (1963) develop this for both modes of operation of their two-chambered system, and Peterson and Sober (1959) give tables for the calculation of the gradient produced by a three or more chambered system, but only where $P_2=2P_1$, $P_3=3P_1$ etc.; this may be termed an "equally additive flow rate" mode of operation (as may any number of chambers in the Varigrad system). Similarly, Niederwieser (1967) gives tables for the calculation of

the gradients produced by 1-8 intercommunicating closed mixing chambers (2-9 chambers); since the flow rate between each chamber is equal in such a case this may be termed an "equal flow rate" mode of operation of the general system.

Smith (1967 - personal communication) was able to produce an algebraic solution for the general case involving three chambers, but this was very complex and Smith stated that it would be difficult, if not impossible, to produce a solution of the general case for four or more chambers.

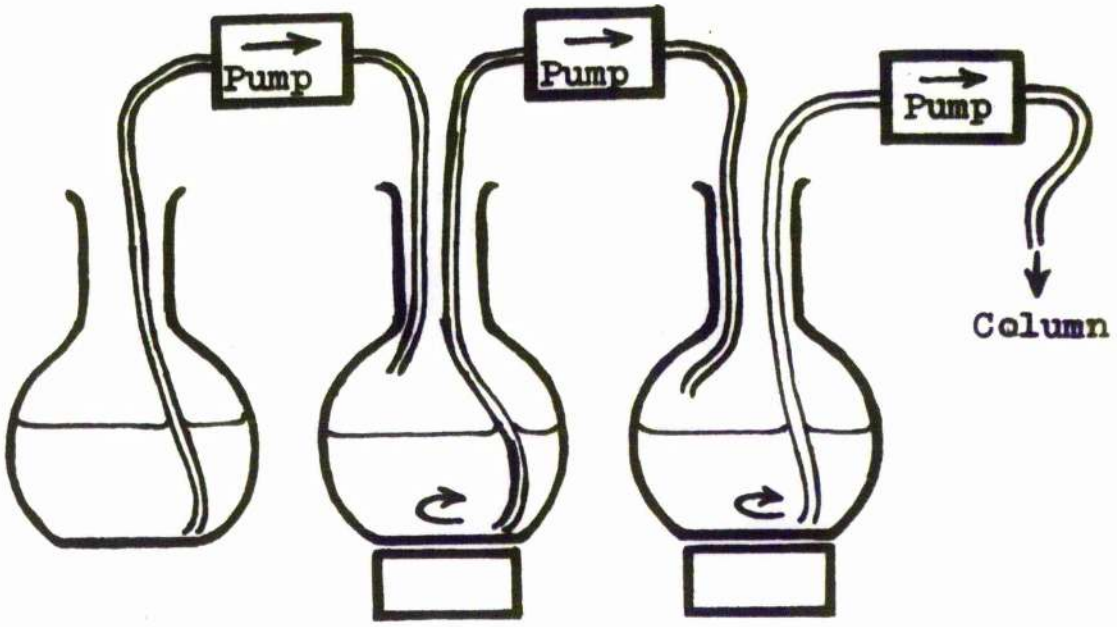
The University's Computer Laboratory was then consulted as to the feasibility of computing the gradients produced by a multichamber system with the chambers connected by pumps as described. Burns, Curtis and Kacsar (1965) have described the use of an analogue computer to simulate the behaviour of the Varigrad, but it seemed doubtful if an analogue computer solution to the general case problem could be easily obtained.

D. Wishart of the Computer Laboratory devised a programme for the University's IBM 1620 II Digital Computer which was capable of producing the theoretical gradient for any set of conditions obtaining in the multichamber system described. This programme was written in Fortran IID and was set up by considering the behaviour of a single chamber in such a system as follows.

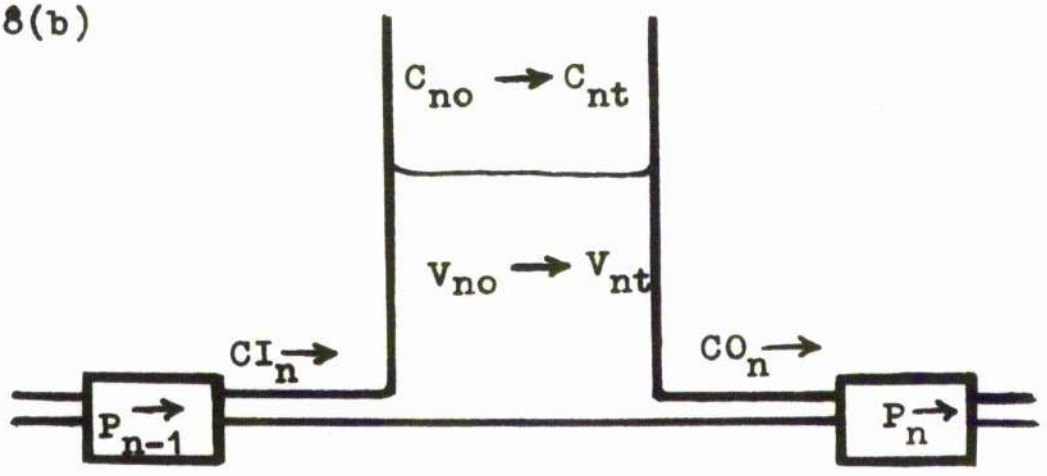
If we consider the n'th chamber of a system (where n can be any number but one) as shown in fig. 8(b), over a

Fig. 8

8(a)



8(b)



n^{th} chamber

very short time interval t, the input concentration to the tank can be assumed to approximate to a constant CI_n and the output to a constant CO_n.

Let the concentration in the chamber at time 0 = C_{no}

Let the concentration in the chamber at time t = C_{nt}

and the volume in the chamber at time 0 = V_{no}

and the pump rates into and out of the chamber be P_{n-1} and P_n respectively.

Assume CO_n = average concentration in n over time t

$= \frac{1}{2}(C_{no} + C_{nt})$

therefore $C_{nt} = 2CO_n - C_{no}$ 1.)

Now mass of solute entering chamber n in time t = $t.CI_n.P_{n-1}$

and mass of solute leaving chamber n in time t = $t.CO_n.P_n$

Initial mass of solute in chamber n at time 0 = $V_{no}.C_{no}$

Final mass = Initial mass + mass entering - mass leaving

$= C_{no}.V_{no} + t.CI_n.P_{n-1} - t.CO_n.P_n$

$= C_{no}.V_{no} + t(CI_n.P_{n-1} - CO_n.P_n)$ 2)

Volume entering chamber n in time t = $P_{n-1}.t$

Volume leaving chamber n in time t = $P_n.t$

Thus volume change = $P_{n-1}.t - P_n.t = (P_{n-1} - P_n)t$

Therefore volume in chamber n at time t

$= V_{no} + (P_{n-1} - P_n)t$ 3)

Concentration in chamber n at time t

$= \frac{\text{Mass of solute at time t}}{\text{Volume at time t}} = C_{nt}$

From 2) and 3)

$$C_{nt} = \frac{C_{no}.V_{no} + t(CI_n.P_{n-1} - CO_n.P_n)}{V_{no} + t(P_{n-1} - P_n)} \qquad 4)$$

and from 1)

$$C_{nt} = 2CO_n - C_{no}$$

Therefore

$$2CO_n - C_{no} = \frac{C_{no}.V_{no} + t(CI_n.P_{n-1} - CO_n.P_n)}{V_{no} + t(P_{n-1} - P_n)}$$

Therefore

$$(2CO_n - C_{no})(V_{no} + t(P_{n-1} - P_n)) = C_{no}V_{no} + t(CI_n.P_{n-1} - CO_n.P_n)$$

Therefore

$$2CO_n(V_{no} + t(P_{n-1} - P_n)) - C_{no}(V_{no} + t(P_{n-1} - P_n)) = C_{no}.V_{no} + t.CI_n.P_{n-1} - t.CO_n.P_n$$

Therefore

$$2CO_n(V_{no} + t(P_{n-1} - P_n)) + tCO_n.P_n = C_{no}.V_{no} + tCI_n.P_{n-1} + C_{no}(V_{no} + t(P_{n-1} - P_n))$$

Therefore

$$CO_n(2V_{no} + 2t(P_{n-1} - P_n) + t.P_n) = 2C_{no}.V_{no} + t.CI_n.P_{n-1} + t.C_{no}(P_{n-1} - P_n)$$

Therefore

$$CO_n(2V_{no} + t(2P_{n-1} - P_n)) = 2C_{no}.V_{no} + t(CI_n.P_{n-1} + C_{no}(P_{n-1} - P_n))$$

Therefore finally

$$CO_n = \frac{2CO_{no}.V_{no} + t(CI_n.P_{n-1} + C_{no}(P_{n-1} - P_n))}{2V_{no} + t(2P_{n-1} - P_n)}$$

If we consider the first chamber in a sequential series of N chambers, the concentration in this reservoir chamber remains constant, hence also the output concentration which is the input concentration CI_2 for chamber two. Provided that the initial conditions in chamber two are known (volume, concentration and input/output pump rates) then CO_2, the output concentration from chamber two can be estimated from equation 5). In the small time interval t this may be regarded as a constant and equal to the input concentration CI_3 for chamber three. Hence the output concentration CO_3 for chamber three can be calculated, and finally the output concentration CO_N from the terminal chamber N, which is the output concentration from the whole system.

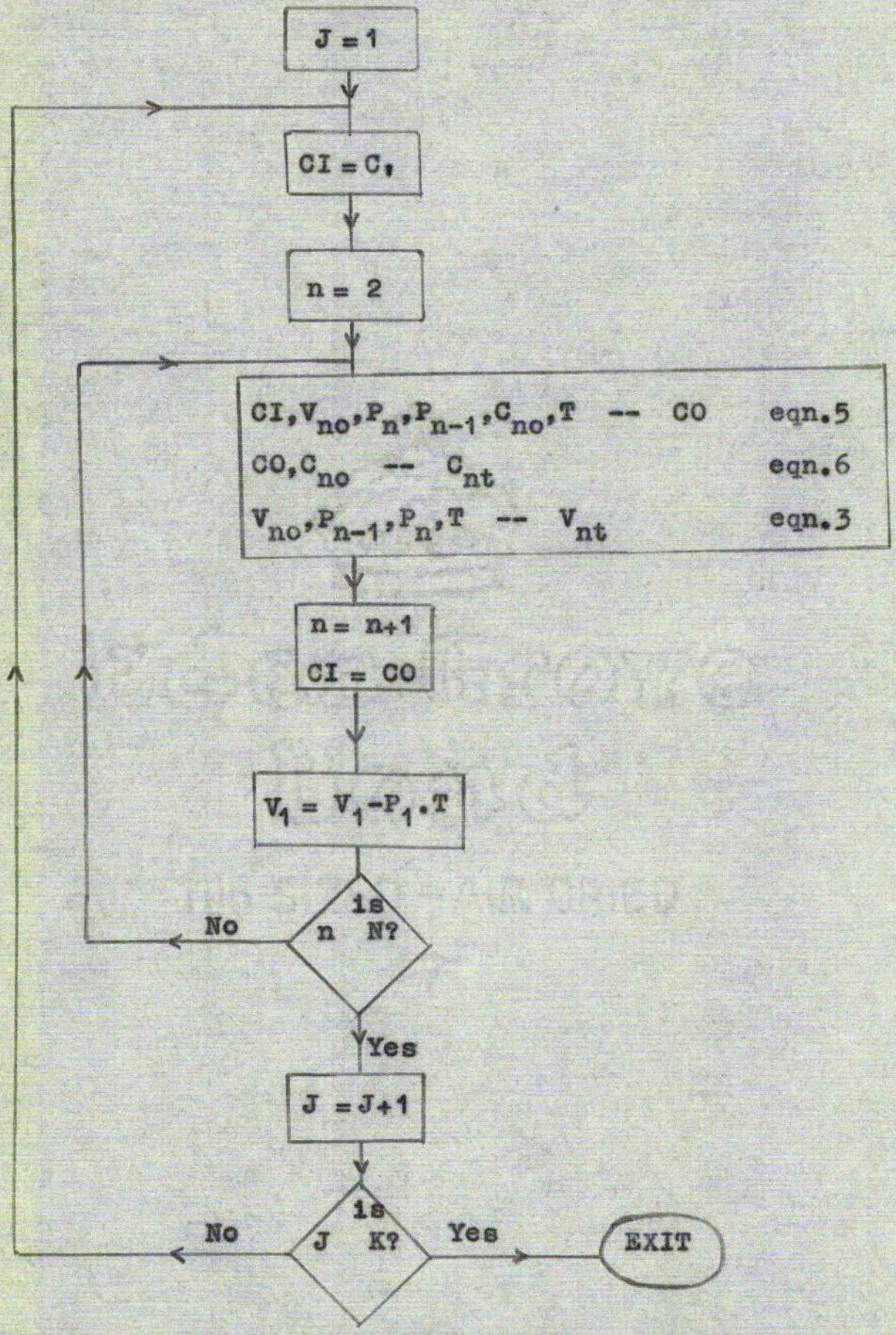
At each stage the concentrations in the chambers after time t may be calculated from equation 1) and the volumes from equation 3). A new time interval from time t to time 2t may then be considered.

The above series of calculations, in the order described, form the basis of the computer programme used in this work. The operation of this programme is summarised in the flow path diagram shown in fig. 9.

The information was fed into the computer as follows: chamber volumes in ml; chamber concentrations as %; pump rates as ml/min. The total experimental time was given in minutes and was divided normally into 100 intervals,

Fig. 9

FLOW DIAGRAM OF COMPUTER PROGRAMME



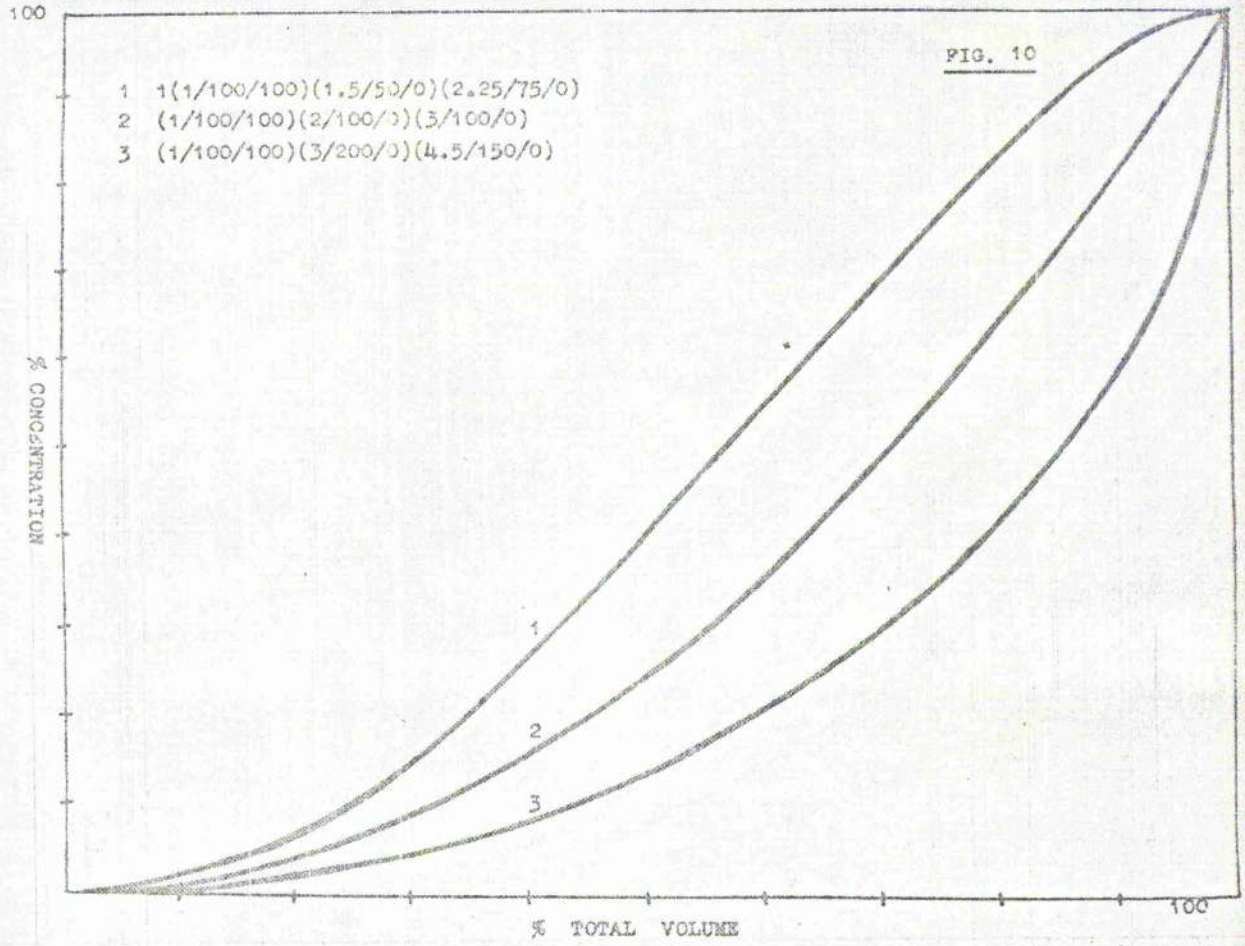
information was printed out after each of these time intervals. These time intervals were independent of the time interval t used in the programme, which in this case was 1/100 minute, and on the flow path diagram in fig. 9, K x t would give the experimental time interval.

For simplicity in describing the conditions in a gradient elution system of this type, a form of shorthand notation was developed to describe each chamber in turn in terms of output pump rate (ml/min), initial volume (ml) and concentration (%) in that order. Thus a chamber with an output of 5ml/min, an initial volume of 200ml and an initial concentration of 50%, would be described by (5/200/50). Where the eluant is more complex than a binary mixture, the concentrations of the various components may be sepcified with respect to a major component of the system e.g. in an aqueous system this would be water, in a non-aqueous system it would probably be the initial eluant applied to the column. Thus, in the above case, if the initial concentrations of minor components A and B were 10% and 20% respectively, the chamber would be described by (5/200/10A:20B). Finally, the chambers are described in order, starting from the "reservoir" end, or the chamber furthest from the column, and where several consecutive chambers are identical (as in an equal flow rate system with chambers containing equal volumes) only one chamber description need be given, followed by a suffix denoting the number of identical chambers thus: $(1/500/100)(1/100/0)_4$. This shorthand

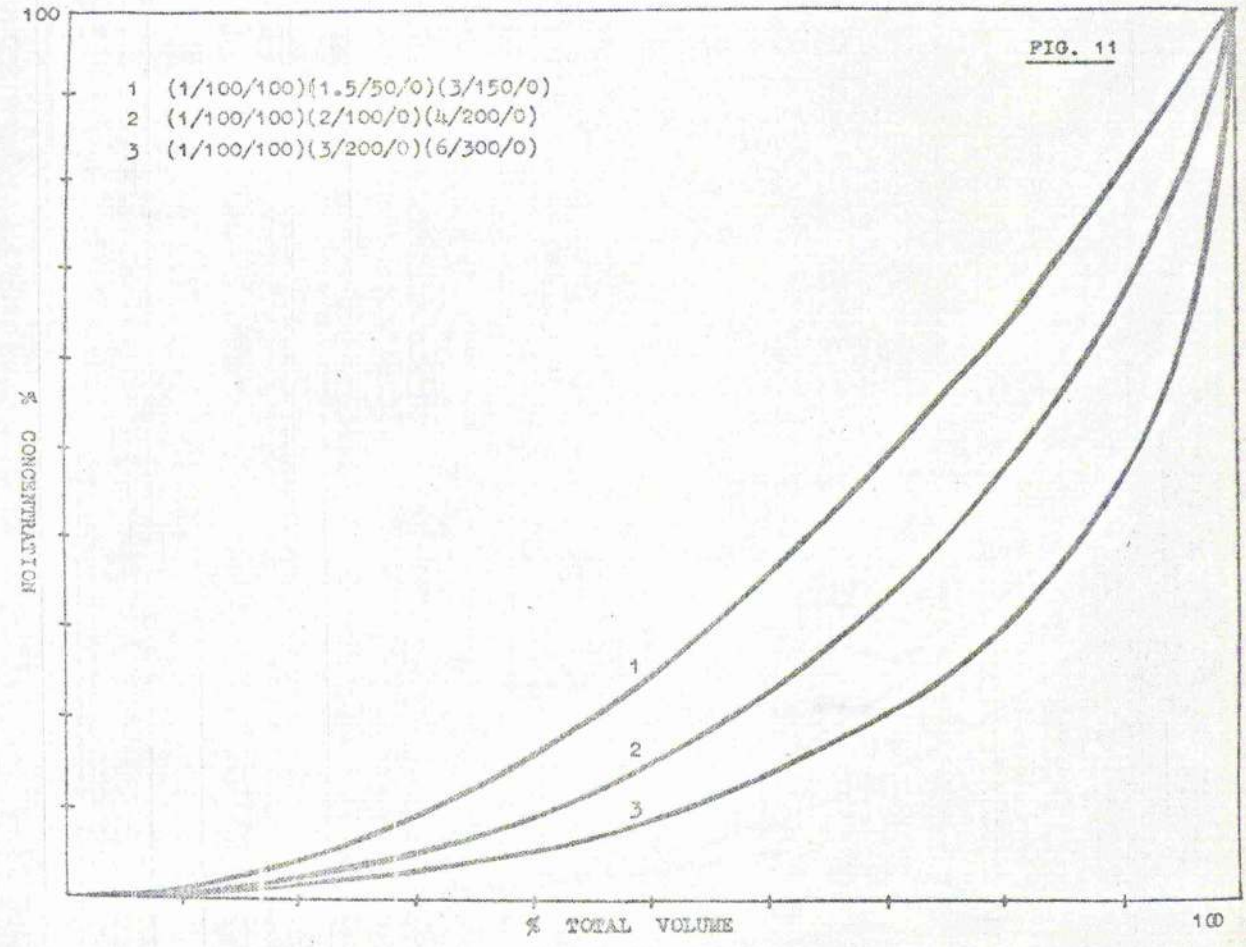
notation is used to describe the gradients shown in figs. 10 -27.

Figs. 10 - 12 show the gradients produced by various flow rate ratios in a 3-chambered system with the volumes adjusted to give a "perfect" system i.e. one in which all the chambers empty simultaneously. Figs. 13 and 14 show the gradients produced by a 3-chambered imperfect system where the input and output flow rates for one chamber are equal and fig. 15 shows the special case where all the flow rates are equal. As can be seen, these latter gradients approximate fairly closely to a straight line, after about 20% of the elution time. Schwab, Rieman and Vaughan (1957) pointed out the value of a 3-chambered constant volume device for the production of linear gradients, although they suggested that the initial reservoir concentration should be twice that in the middle chamber and that the mixing chamber volumes should be equal; in such a case, the gradient would be approximately linear even close to the beginning.

Experimental verification of the computer results was obtained for several of these 3-chambered systems. The gradients were set up using D.C.L. Series III pumps, with conical flasks as reservoirs. Connections were made with fine plastic tubing, and mixing was by means of magnetic stirrers. A gradient of potassium dichromate in water was used, and for convenience this was monitored by means of its optical density at 245nm using the LKB Uvicord used


100
% CONCENTRATION
1 1(1/100/100)(1.5/50/0)(2.25/75/0)
2 (1/100/100)(2/100/0)(3/100/0)
3 (1/100/100)(3/200/0)(4.5/150/0)
1
2
3
100
% TOTAL VOLUME

PIG. 10


100
% CONCENTRATION
1 (1/100/100)(1.5/50/0)(3/150/0)
2 (1/100/100)(2/100/0)(4/200/0)
3 (1/100/100)(3/200/0)(6/300/0)
1
2
3
100
% TOTAL VOLUME

PIG. 11

FIG. 12

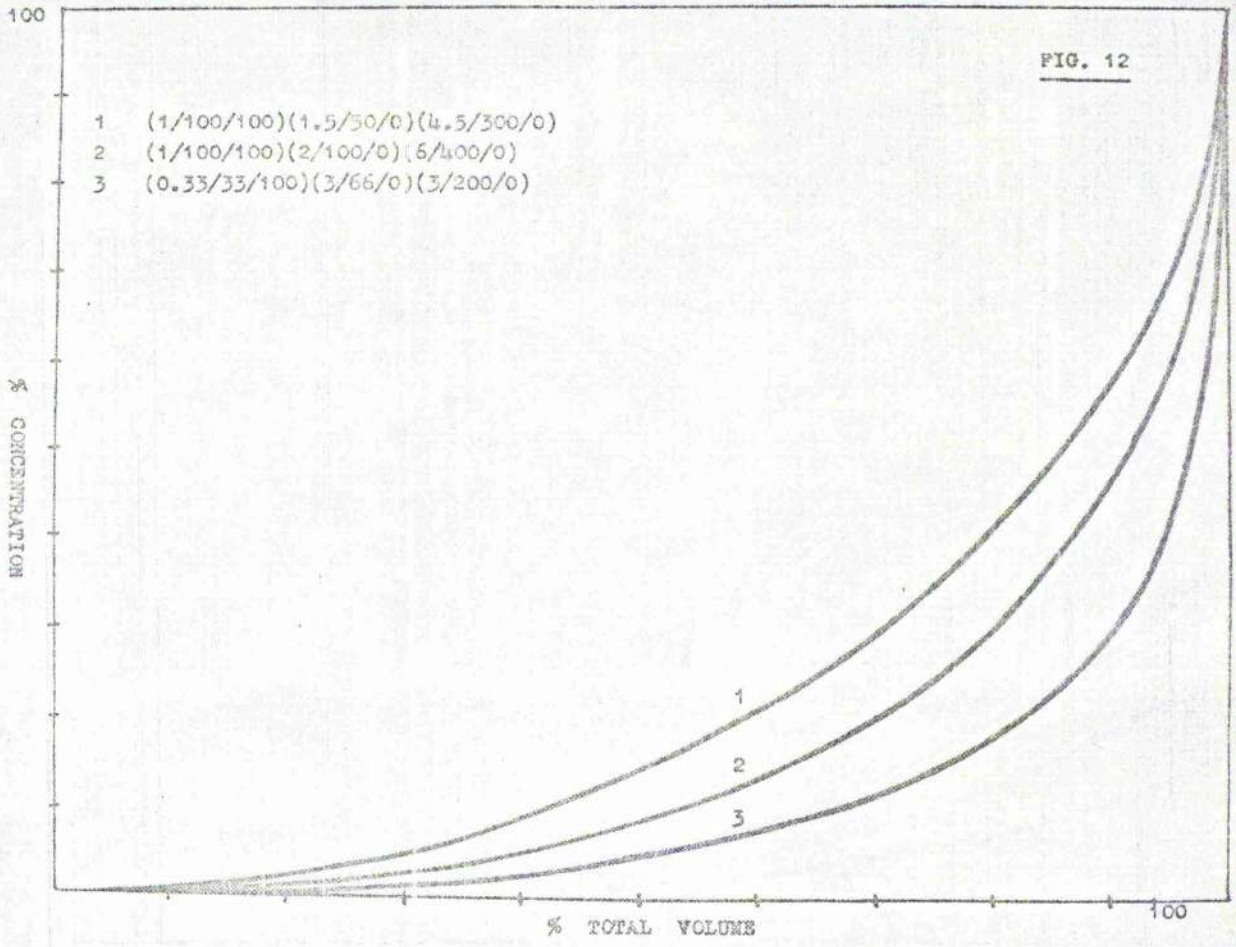
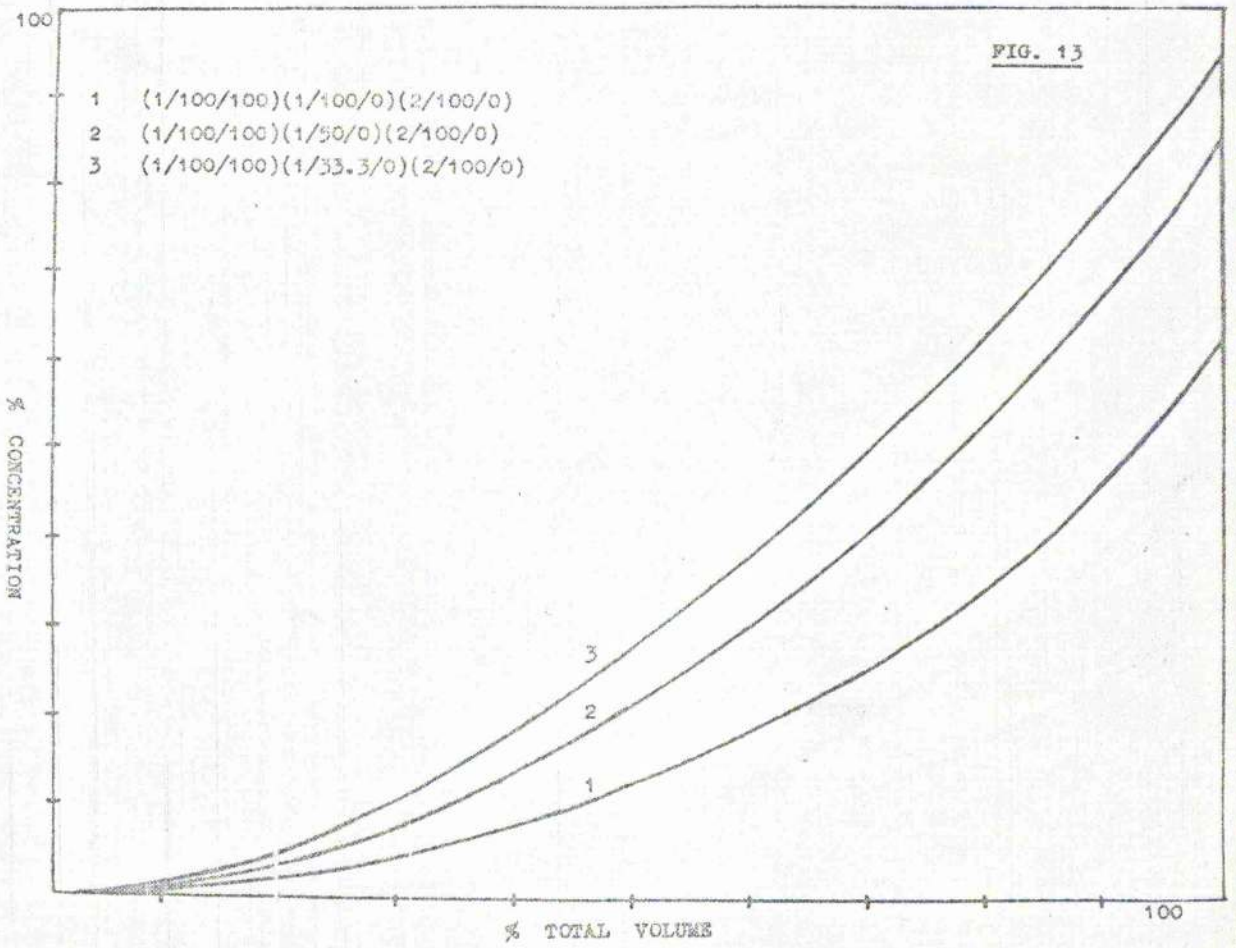

100
1 (1/100/100)(1.5/50/0)(4.5/300/0)
2 (1/100/100)(2/100/0)(6/400/0)
3 (0.33/33/100)(3/66/0)(3/200/0)
% CONCENTRATION
1
2
3
100
% TOTAL VOLUME

FIG. 13


100
1 (1/100/100)(1/100/0)(2/100/0)
2 (1/100/100)(1/50/0)(2/100/0)
3 (1/100/100)(1/33.3/0)(2/100/0)
% CONCENTRATION
3
2
1
100
% TOTAL VOLUME

for the gradient elution chromatography of the plant quinones (see the Experimental Section). It was found that a concentration of .0083% AnalaR potassium dichromate in distilled water gave an optical density of 1.0 in the Uvicord cell. As an initial check of the system, a linear gradient was set up using two chambers such that the output from the mixing chamber was twice the input. The optical density was plotted using the Vitatron recorder as a log recorder and a straight line plot of optical density against time was obtained.

The gradients produced by a 3-chambered system were in excellent agreement with those produced by the Computer. Further verification was also obtained by comparison of fig. 10, curve 3 with the 3-chambered system of Peterson and Sober (1959), these too, give identical curves. Fig. 16 gives the computed gradients for the Varigrad and again comparison with Peterson and Sober (1959) shows complete agreement. Fig. 17 shows the effect of doubling the flow rate out of a single chamber in the 9-chambered Varigrad system and doubling the volume in that chamber to compensate. Such an effect could be produced by doubling the cross sectional area of a chamber in the Varigrad itself. It can be seen that in contrast to the equally additive operation of the Varigrad, the curves in fig. 17 are not symmetrical about the 50% elution mark. Since the general case, using pump interconnected chambers, should be more versatile than either the Varigrad or the closed multichamber

FIG. 14

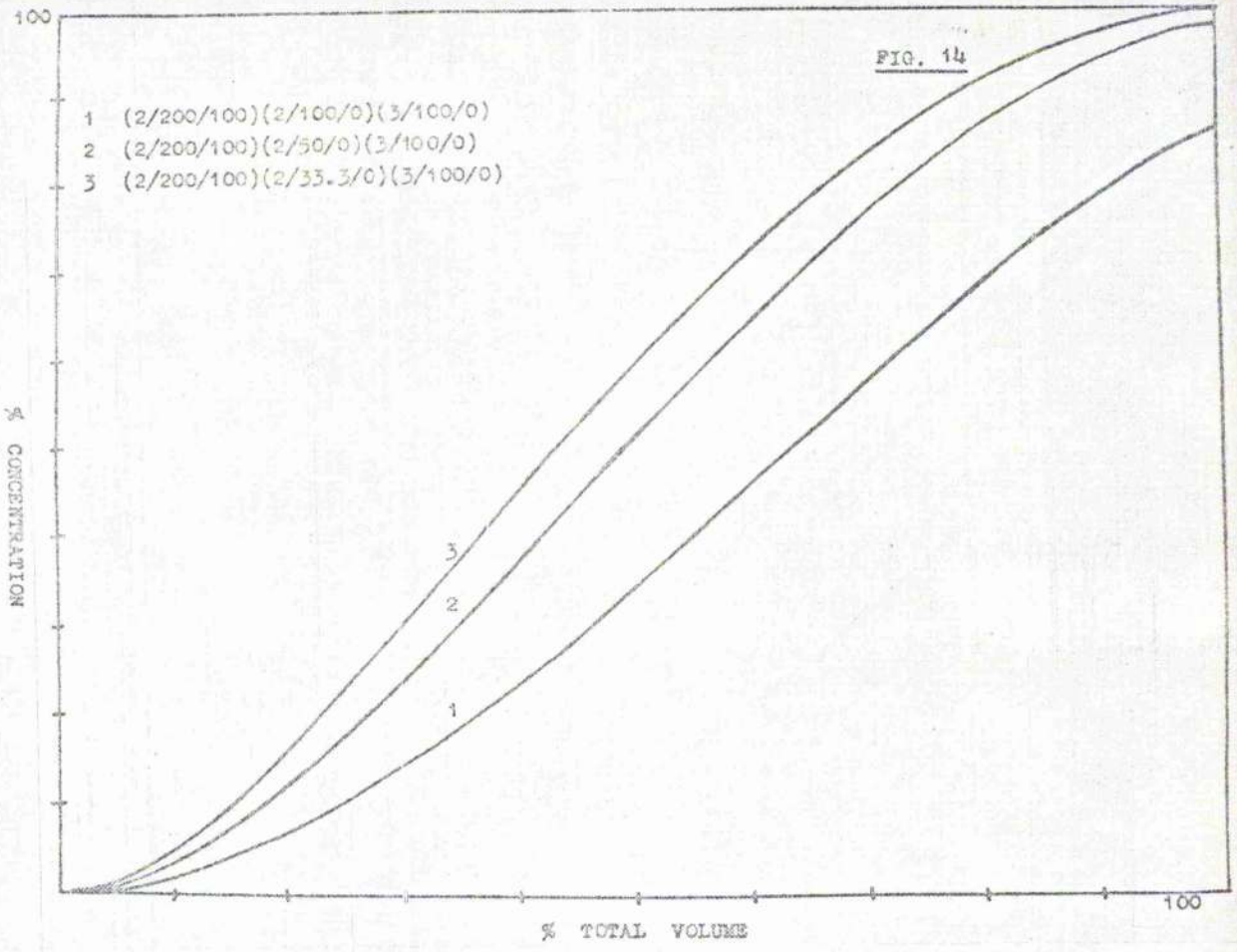
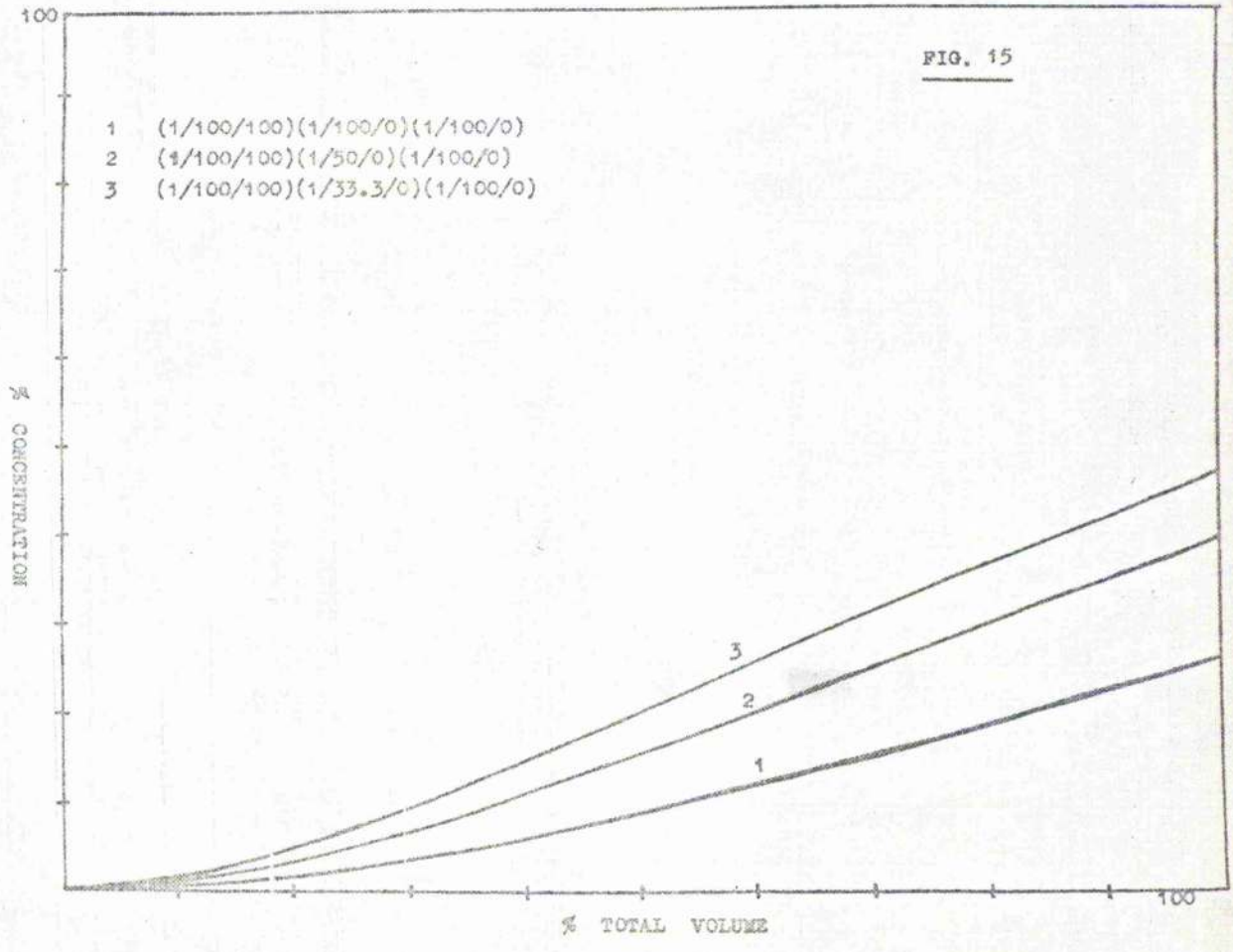

100
1 (2/200/100)(2/100/0)(3/100/0)
2 (2/200/100)(2/50/0)(3/100/0)
3 (2/200/100)(2/33.3/0)(3/100/0)
% CONCENTRATION
3
2
1
100
% TOTAL VOLUME

FIG. 15


100
1 (1/100/100)(1/100/0)(1/100/0)
2 (1/100/100)(1/50/0)(1/100/0)
3 (1/100/100)(1/33.3/0)(1/100/0)
% CONCENTRATION
3
2
1
100
% TOTAL VOLUME

type, it should be possible to obtain equivalent results using a smaller number of chambers, consequently the behaviour of a 5-chambered system was examined. Figs. 18-21 show a variety of the gradients produced by such a system when operating in a "perfect" manner i.e. so that all the chambers empty simulataneously at the end of a run. Fig. 18 shows the chamber contributions when the system is operating in the "equally additive flow rate" mode of operation of the Varigrad. Figs. 19, 20 and 21 respectively show the effect of halving, doubling and trebling the volume and output of a chamber in a 5-chambered additive flow rate perfect system. It can be seen that sufficient flexibility could probably be obtained by either halving or doubling a chamber output and volume. Figs. 22 and 23 show the effect on the gradient of doubling the volume and output of passive chambers in the system. From these it can be seen that increasing the volume and output of a passive chamber beyond the contributing one tends to reduce its overall contribution. Increasing the output and volume for a passive chamber ahead of the contributing chamber tends to reduce its initial effect only slightly, whilst depressing its later contribution to a greater extent.

Figs. 24-27 show the behaviour of a 5-chambered system operating in an equal flow rate mode of operation. This mode of operation is described by Niederwieser (1967) for equal chamber volumes and may be useful as it is easily

FIG. 16

1 (1/100/0)(2/100/0)(3/100/0)(4/100/0)(5/100/0)(6/100/0)(7/100/0)(8/100/0)(9/100/100)
2 " " " " " " " (8/100/100)(9/100/0)
3 " " " " " " (7/100/100)(8/100/0) "
4 " " " " " (6/100/100)(7/100/0) " "
5 " " " " (5/100/100)(6/100/0) " " "
6 " " " (4/100/100)(5/100/0) " " " "
7 " " (3/100/100)(4/100/0) " " " " "
8 " (2/100/100)(3/100/0) " " " " " "
9 (1/100/100)(2/100/0) " " " " " " "

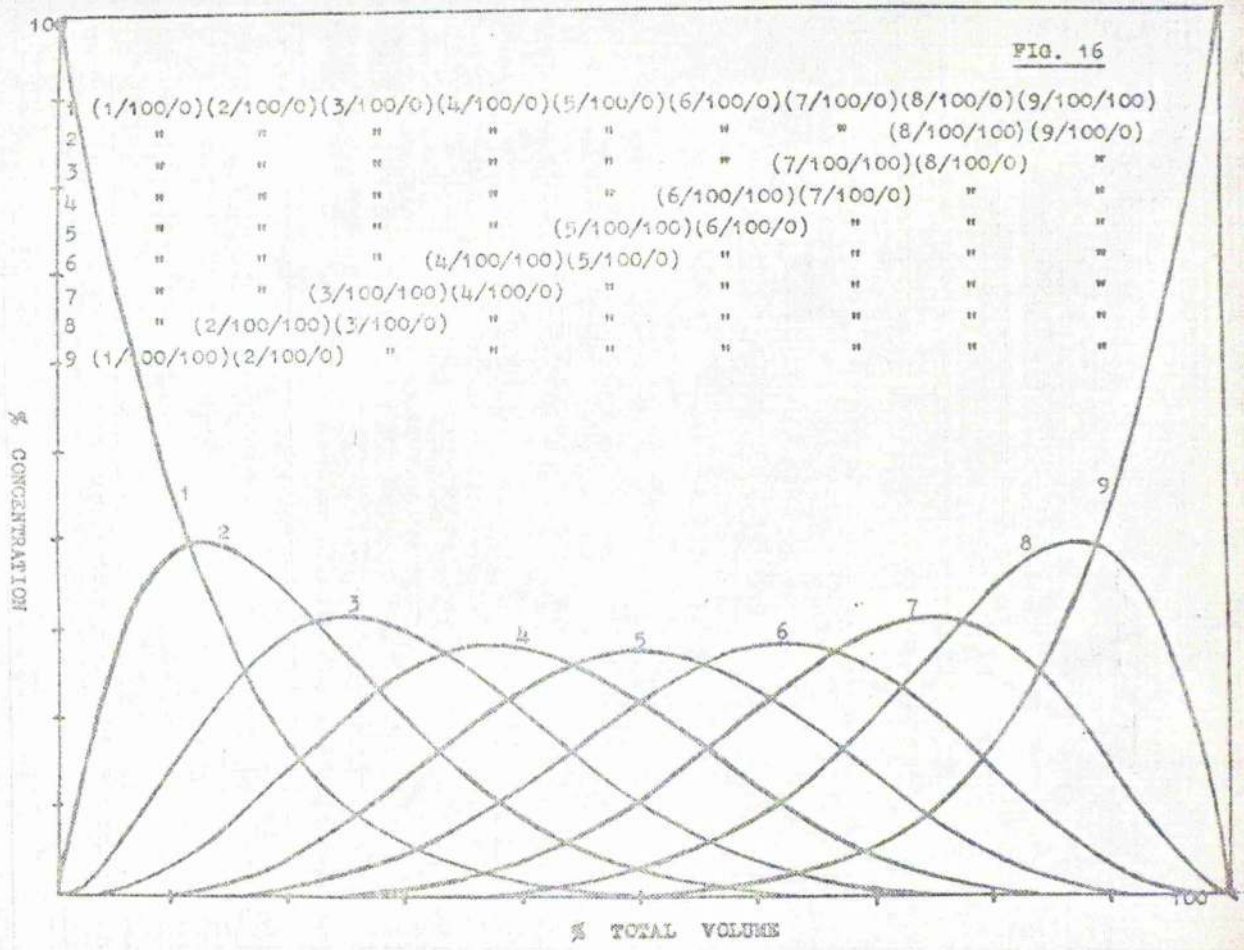


FIG. 17

1 (1/100/0)(2/100/0)(3/100/0)(4/100/0)(5/100/0)(6/100/0)(7/100/0)(8/100/0)(10/200/100)
2 " " " " " " " (9/200/100)(10/100/0)
3 " " " " " " (8/200/100)(9/100/0) "
4 " " " " " (7/200/100)(8/100/0) " "
5 " " " " (6/200/100)(7/100/0) " " "
6 " " " (5/200/100)(6/100/0) " " " "
7 " " (4/200/100)(5/100/0) " " " " "
8 " (3/200/100)(4/100/0) " " " " " "
9 (2/200/100)(3/100/0) " " " " " " "

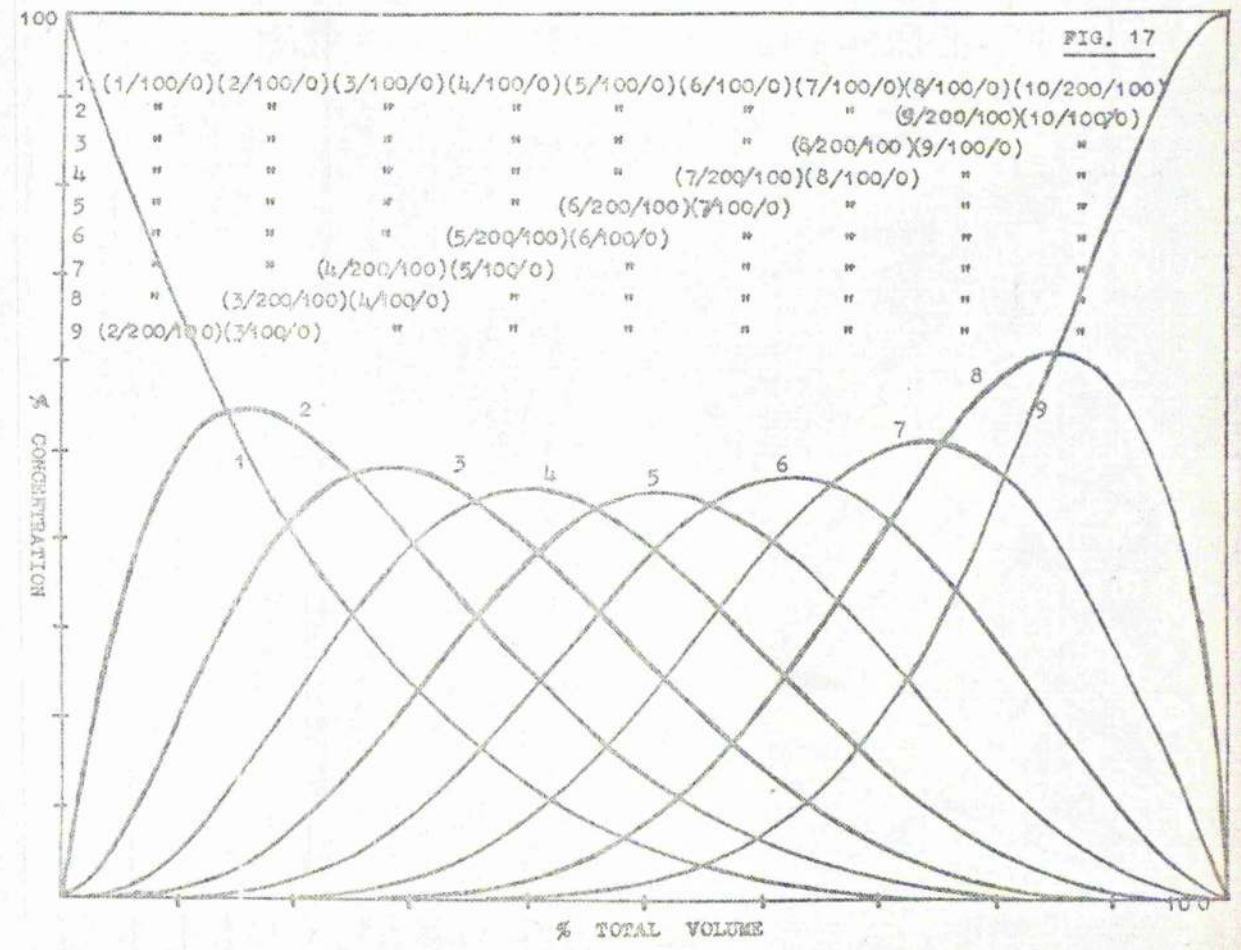


FIG. 18

1 (1/100/0)(2/100/0)(3/100/0)(4/100/0)(5/100/100)
2 " " " (4/100/100)(5/100/0)
3 " " (3/100/100)(4/100/0) "
4 " (2/100/100)(3/100/0) " "
5 (1/100/100)(2/100/0) " " "

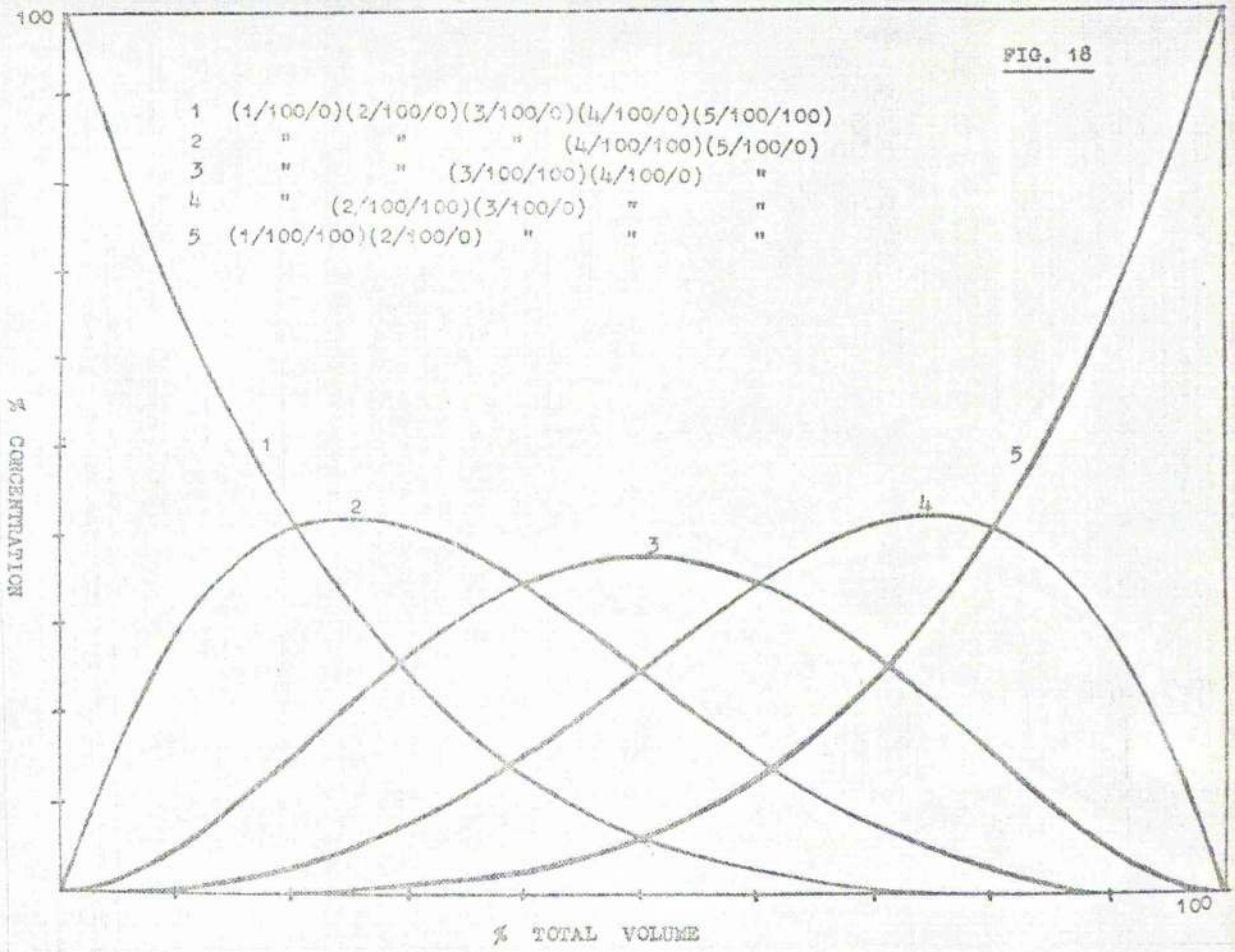
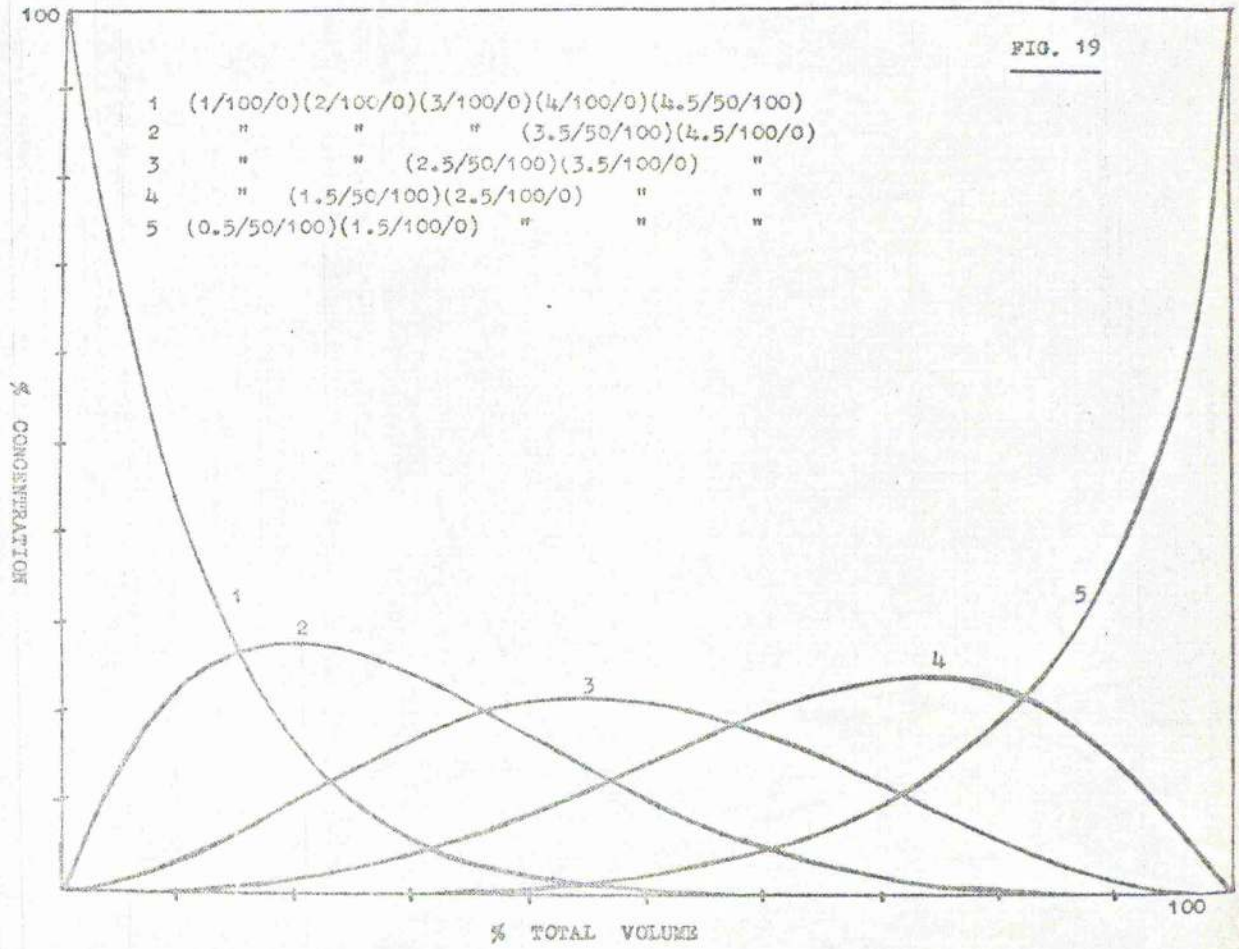


FIG. 19

1 (1/100/0)(2/100/0)(3/100/0)(4/100/0)(4.5/50/100)
2 " " " (3.5/50/100)(4.5/100/0)
3 " " (2.5/50/100)(3.5/100/0) "
4 " (1.5/50/100)(2.5/100/0) " "
5 (0.5/50/100)(1.5/100/0) " " "



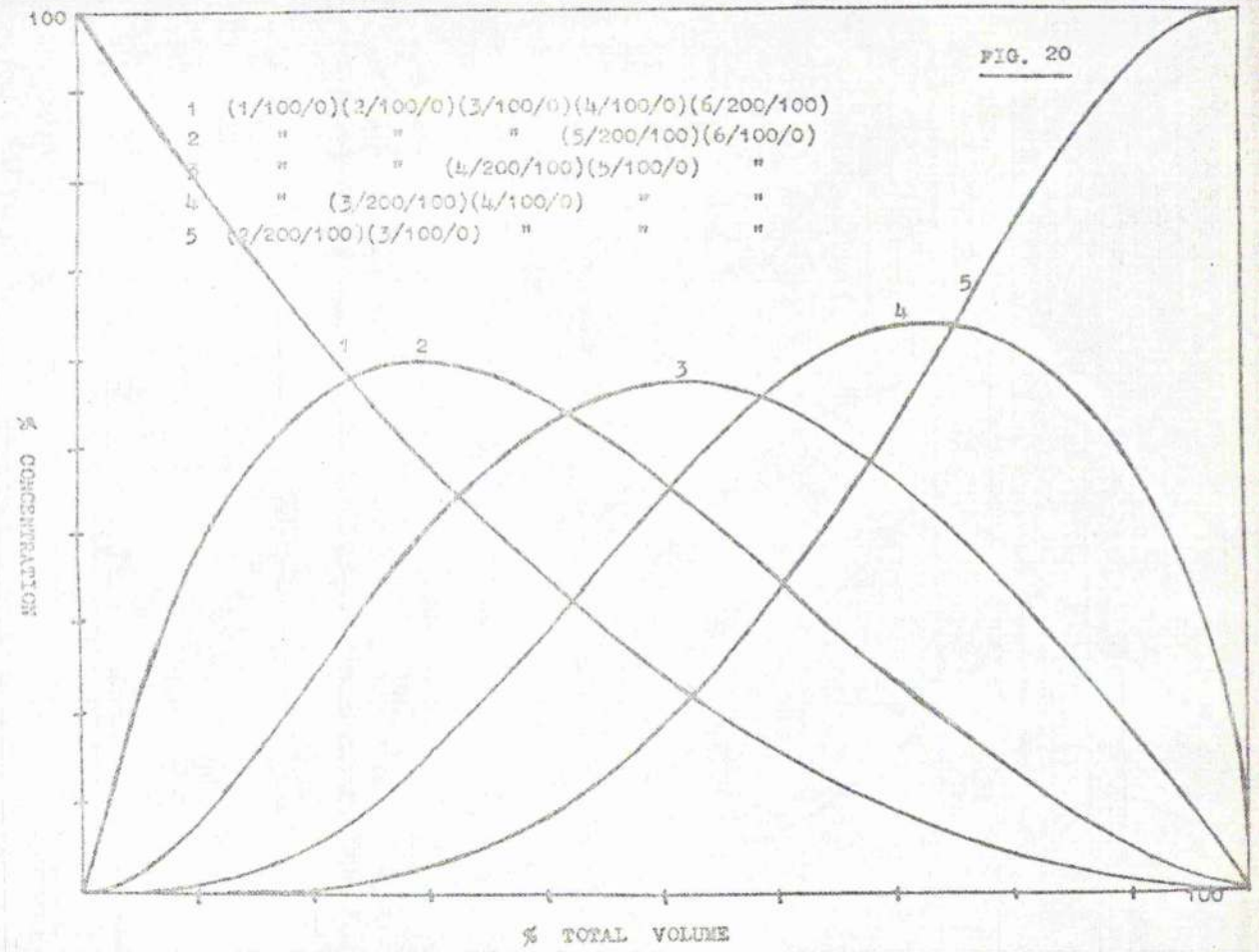

100
1 (1/100/0)(2/100/0)(3/100/0)(4/100/0)(6/200/100)
2 " " " (5/200/100)(6/100/0)
3 " " (4/200/100)(5/100/0) "
4 " (3/200/100)(4/100/0) " "
5 (2/200/100)(3/100/0) " " "
1
2
3
4
5
% CONCENTRATION
100
% TOTAL VOLUME

FIG. 20

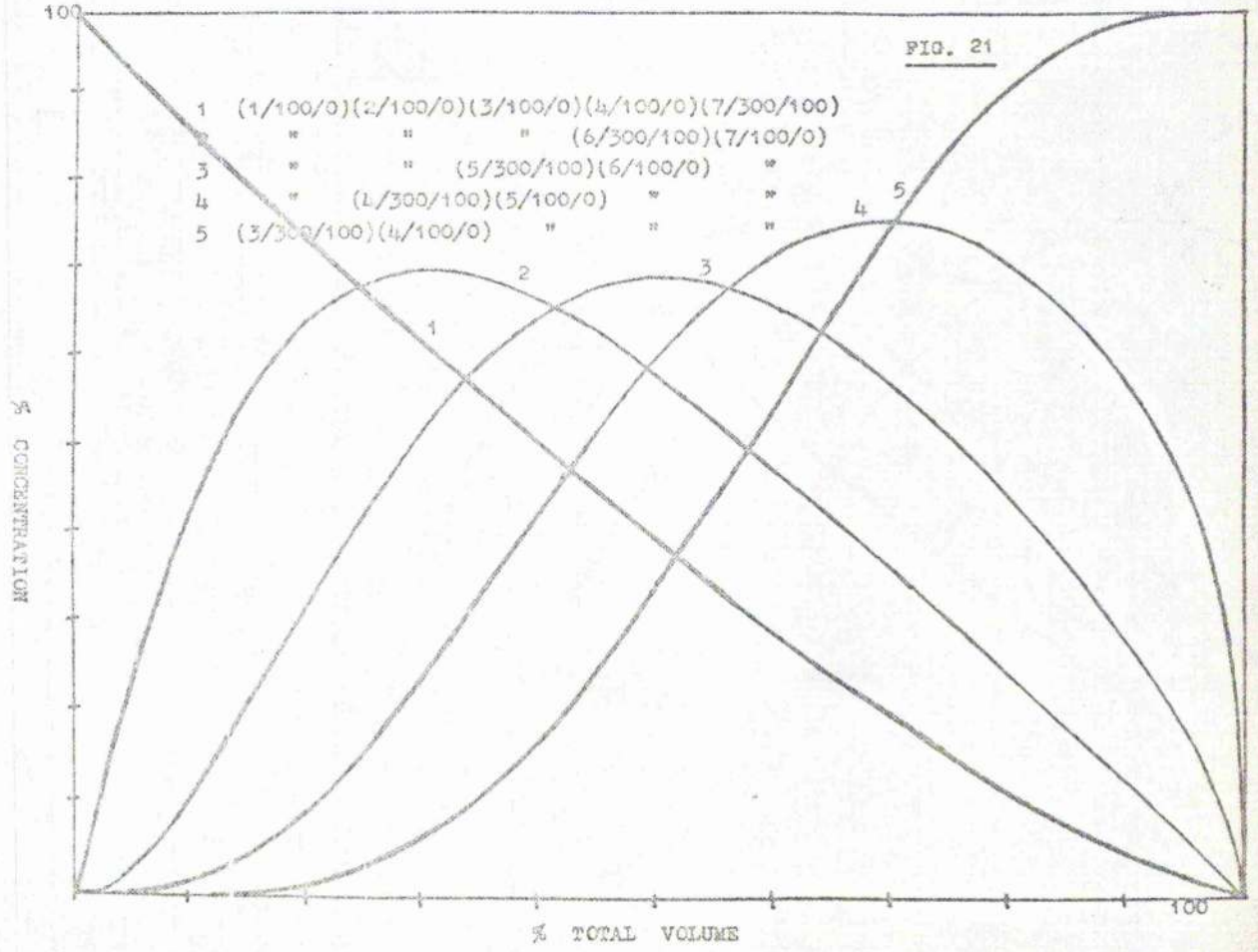

100
1 (1/100/0)(2/100/0)(3/100/0)(4/100/0)(7/300/100)
2 " " " (6/300/100)(7/100/0)
3 " " (5/300/100)(6/100/0) "
4 " (1/300/100)(5/100/0) " "
5 (3/300/100)(4/100/0) " " "
1
2
3
4
5
% CONCENTRATION
100
% TOTAL VOLUME

FIG. 21

FIG. 22

1 (1/100/0)(2/100/0)(4/200/0)(5/100/100)(6/100/0)
2 " (3/200/0)(4/100/100)(5/100/0) "
3 " (2/100/0)(3/100/100)(5/200/0) "
4 " (2/100/100)(4/200/0)(5/100/0) "

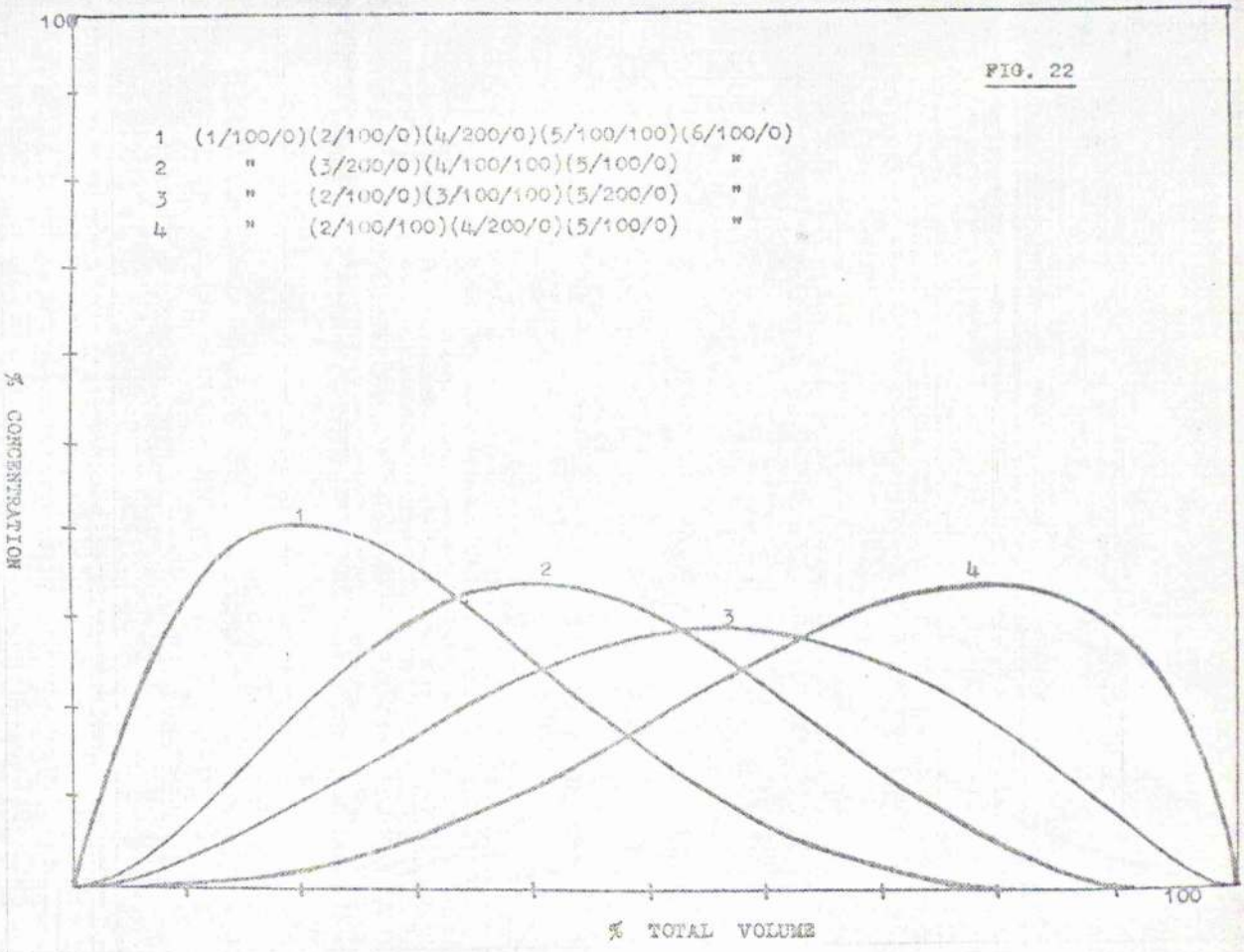
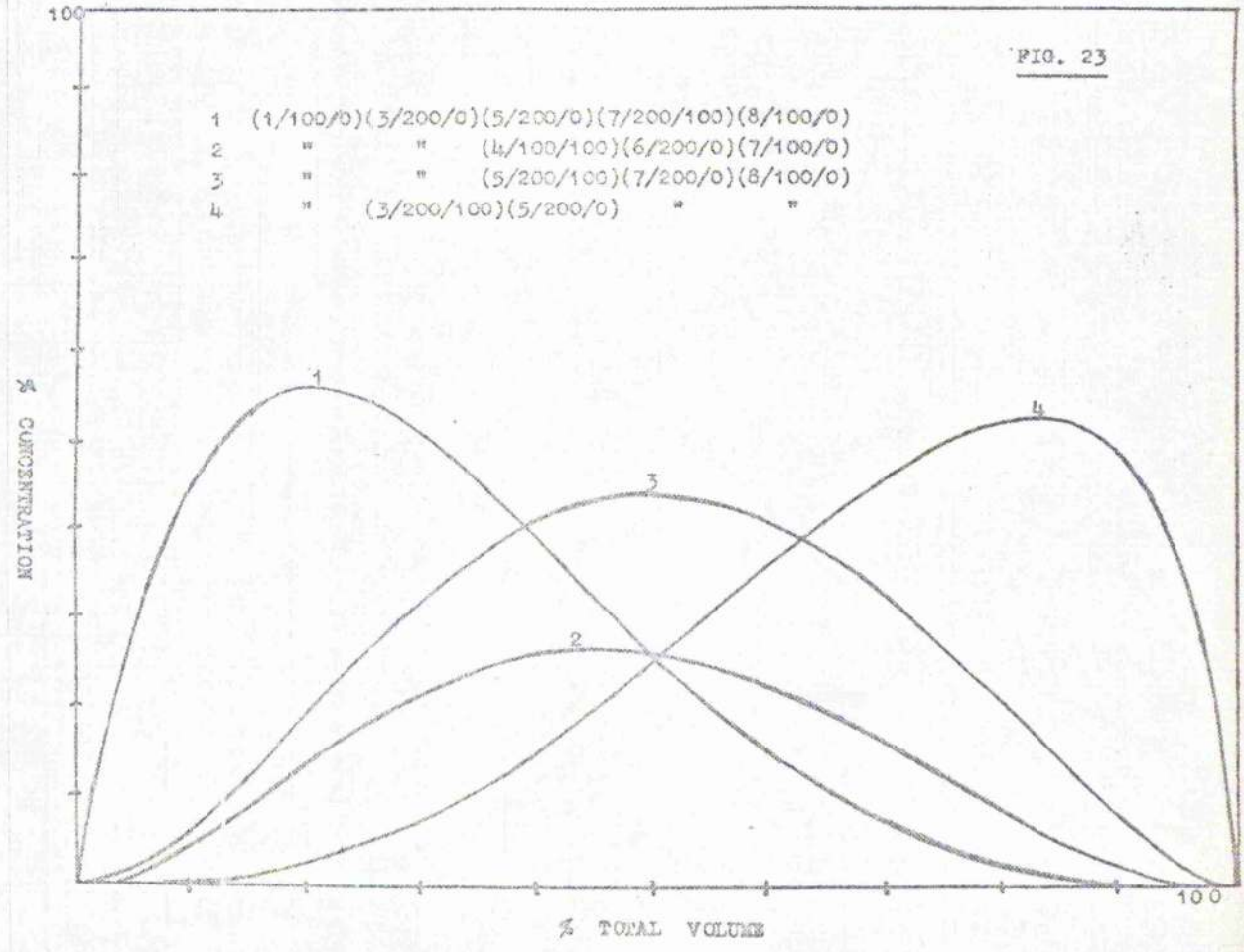


FIG. 23

1 (1/100/0)(3/200/0)(5/200/0)(7/200/100)(8/100/0)
2 " " (4/100/100)(6/200/0)(7/100/0)
3 " " (5/200/100)(7/200/0)(8/100/0)
4 " (3/200/100)(5/200/0) " "



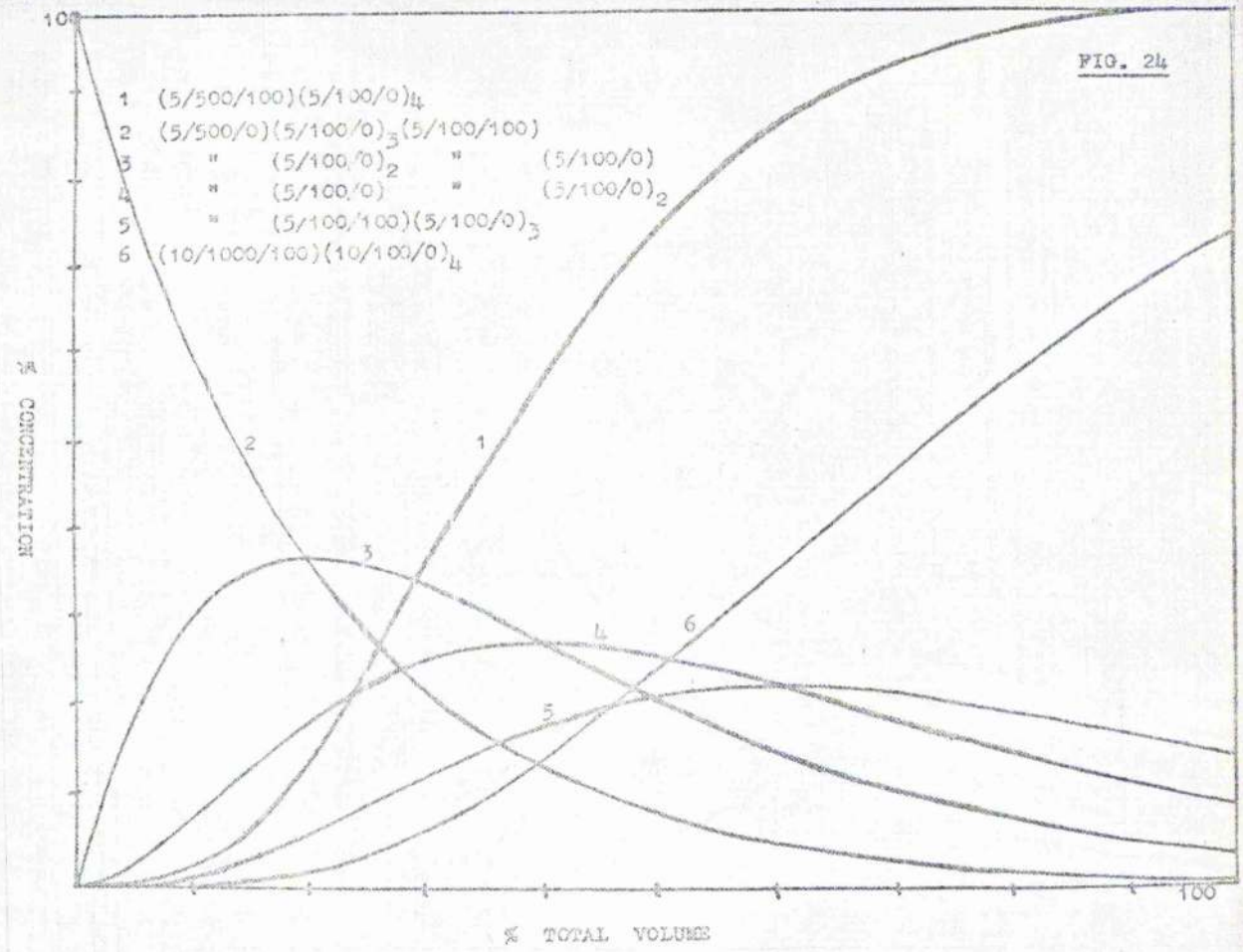

100
1 (5/500/100)(5/100/0)4
2 (5/500/0)(5/100/0)3(5/100/100)
3 " (5/100/0)2 " (5/100/0)
4 " (5/100/0) " (5/100/0)2
5 " (5/100/100)(5/100/0)3
6 (10/1000/100)(10/100/0)4
% CONCENTRATION
% TOTAL VOLUME
100

FIG. 24

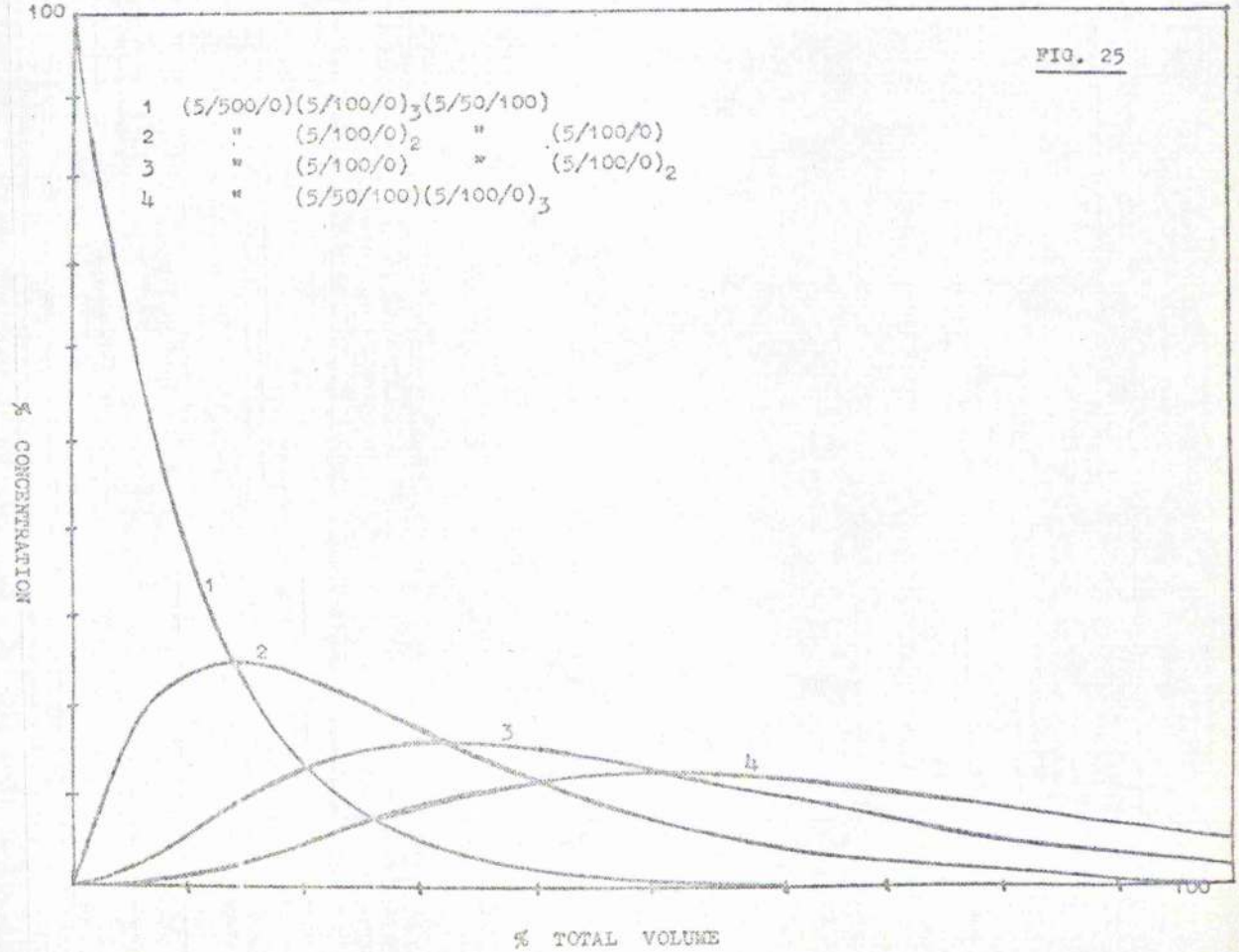

100
1 (5/500/0)(5/100/0)3(5/50/100)
2 " (5/100/0)2 " (5/100/0)
3 " (5/100/0) " (5/100/0)2
4 " (5/50/100)(5/100/0)3
% CONCENTRATION
% TOTAL VOLUME
100

FIG. 25

FIG. 27

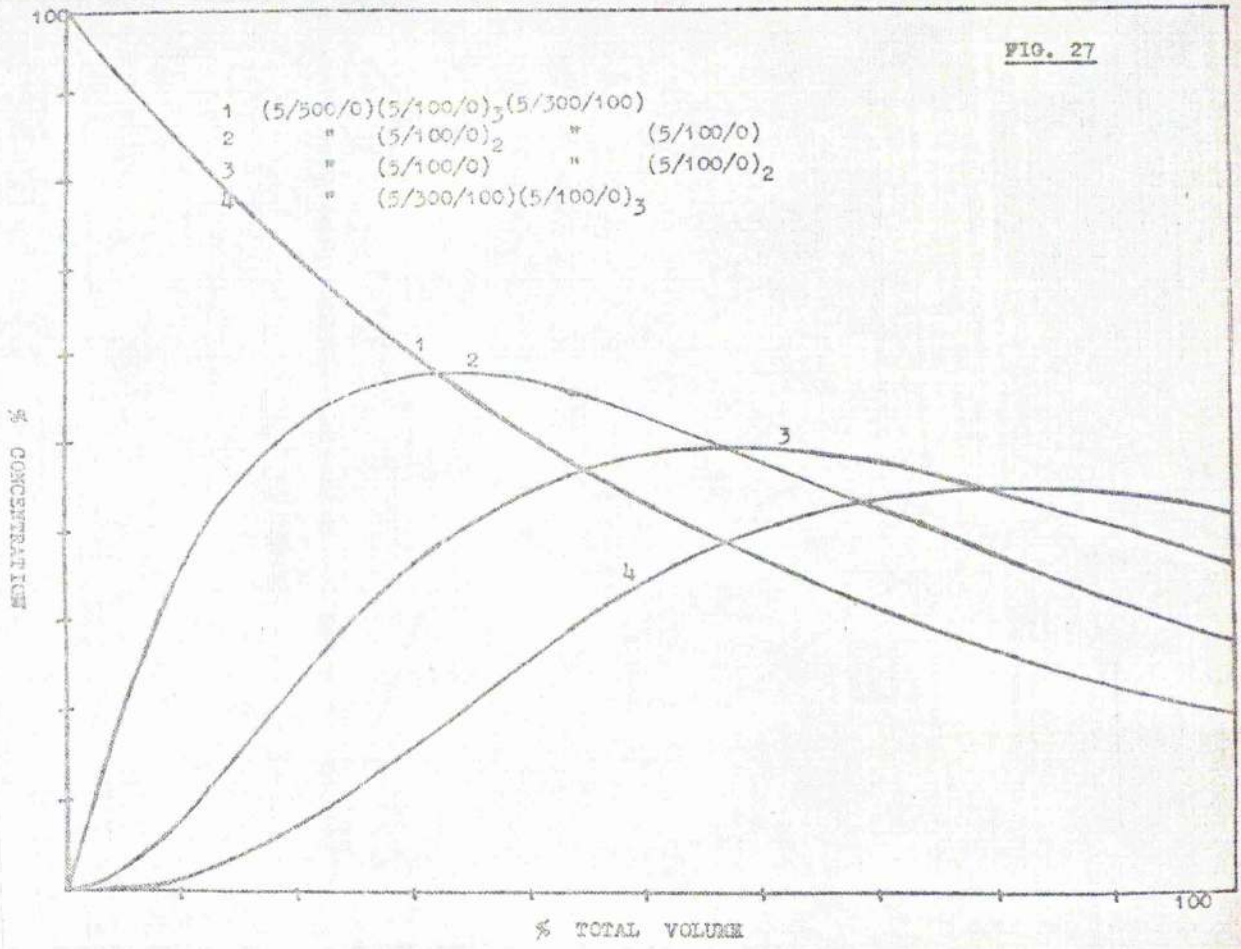
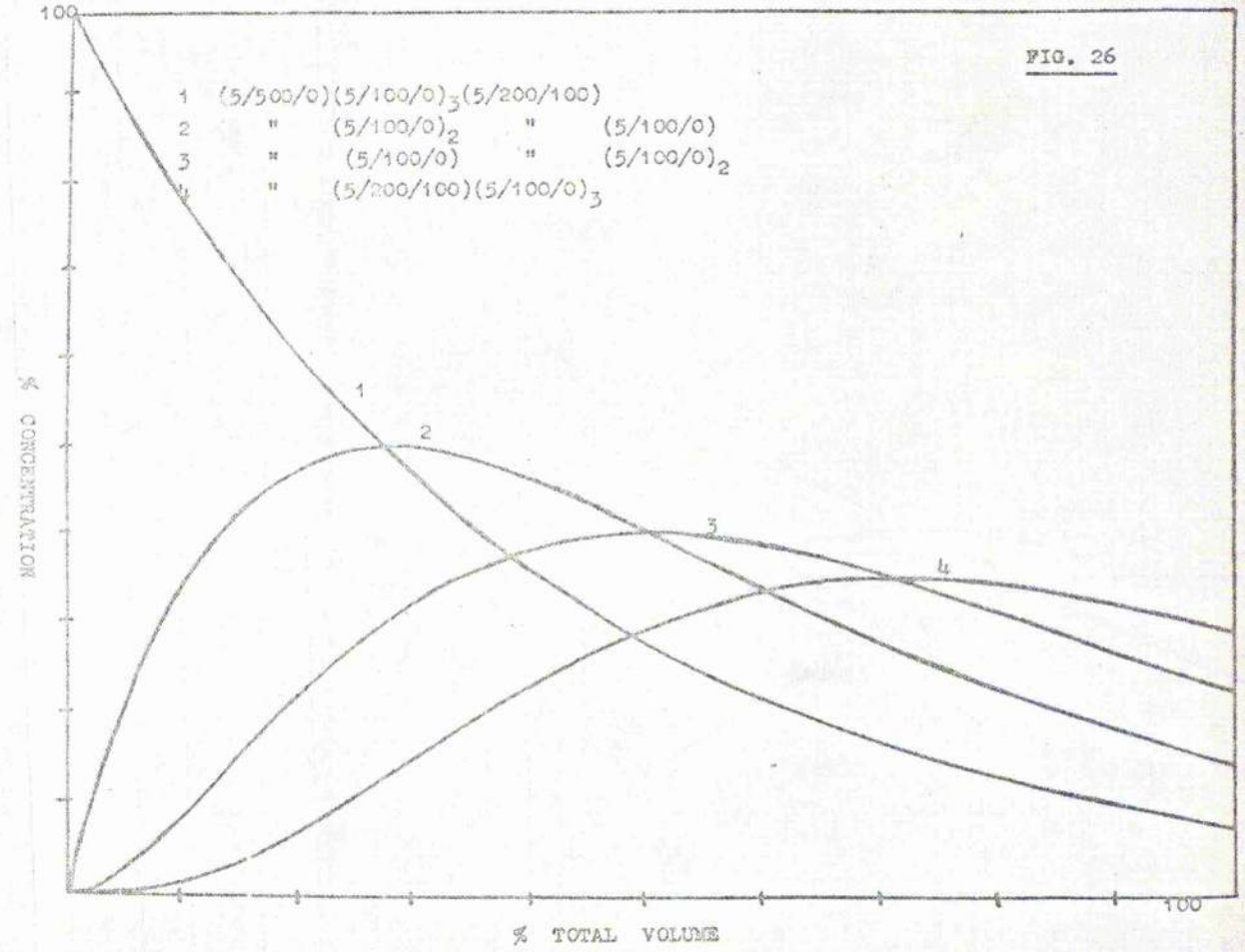

100
1 (5/500/0)(5/100/0)3(5/300/100)
2 " (5/100/0)2 " (5/100/0)
3 " (5/100/0) " (5/100/0)2
4 " (5/300/100)(5/100/0)3
1
2
3
4
% CONCENTRATION
100
% TOTAL VOLUME

FIG. 26


100
1 (5/500/0)(5/100/0)3(5/200/100)
2 " (5/100/0)2 " (5/100/0)
3 " (5/100/0) " (5/100/0)2
4 " (5/200/100)(5/100/0)3
1
2
3
4
% CONCENTRATION
100
% TOTAL VOLUME

produced by a multichannel peristaltic pump. Fig. 24 shows the chamber contributions for equal chamber volumes and the three following figures show the effect of halving, doubling and trebling the active chamber volumes. A comparison of curve 1 and curve 6 in fig. 24 shows the effect of doubling the pump rate or, alternatively, of doubling the elution time. It can be seen that the curves of the gradients produced by this mode of operation are not as sharp as those produced by the previous mode, thus less complex gradients can be produced. This effect can also be seen by comparing the gradients of Chase (1963) and Niederwieser (1967) with those produced by the Varigrad.

Summary

A system is described for the production of complex concentration gradients for use in gradient elution chromatography. This is a sequential multichamber system utilising constant speed pumps to connect the stirred mixing chambers and as such, is resistant to organic solvents and is unaffected by density differences. Although designed for binary solvent mixtures, the system can be used to produce multi-component gradients as suggested by Larrebee and Klingman (1963). The flexibility of this system is such that it can duplicate the action of the Varigrad of Peterson and Sober (1959) and the constant volume device described by Svensson and Forchheimer (1962), Chase (1963)

and Niederwieser (1967) as well as operating in other different ways. A computer programme has been derived for this system which is generally applicable to any gradient elution system in which the flow rates remain constant throughout a complete operation. Chamber contributions for various different conditions obtaining in such a system have been presented.

EXPERIMENTAL SECTION

EXPERIMENTAL (A)

PREPARATION OF LIPID EXTRACTS OF LEAVES

1) LEAF MATERIAL AND GENERAL PRECAUTIONS

The leaves used during the carotenoid investigations were obtained from growing plants of sugar beet, kale, broccoli, cabbage or brussel sprouts. Some of the quinone investigations were performed on the same leaf material but for most, including the work on seasonal variation, spinach beet (Beta vulgaris L. Cicla) was grown outside and leaves were removed from the growing plant between 9a.m. and 10a.m. These leaves were extracted within one hour of cropping.

For the extraction experiments, seedlings of radish (Raphanus sativus var. Crimson Giant) were grown as described in Experimental Section (D). The leaves of horse chestnut (Aesculus hippocastanum) used for the isolation of plastoquinone-4 (Experimental Section E) were obtained from mature trees growing in the grounds of the University Botanic Gardens.

2) EXTRACTION OF LEAF MATERIAL

Method a)

Initially the extraction of fresh leaf material was performed by a modification of the method advocated by many workers (e.g. Booth, 1957). This method involves the grinding of the leaf material with sand and organic solvents with the aid of a mortar and pestle. It was fairly

successfully used for both large-scale (300-600g of leaves) and small-scale (10-30g of leaves) extractions, but method b) below was found to be more convenient for small-scale extractions in later work.

The fresh leaves were stripped of their mid-ribs, cut into sections of 2-3cm square and ground rapidly in portions (only in the case of large-scale extractions) in a mortar and pestle, with about one and a half times their own weight of sand and sufficient acetone to cover the mixture. The acetone extract was decanted off, filtered through cotton wool and the residue re-extracted in a similar manner. After the second acetone extraction the residue was similarly extracted with either n-hexane or 60-80 petroleum ether (which consists mainly of n-hexane). The hexane extract was decanted off and the residue was re-extracted alternately with acetone and n-hexane (or 60-80 petroleum ether) until no more green colour was extracted.

The combined extracts were then washed with distilled water, to remove acetone and water soluble leaf constituents, and the dark green solution (total volume of approximately 2ml/g of original fresh leaf material) was taken to dryness in a rotary evaporator with a bath temperature of 45°C. Any water present was azeotroped off by the careful addition of small volumes of ethanol. The residue obtained was dissolved in hexane (approximately 1ml/4g of original fresh leaf material) and samples of this solution were subjected

to saponification and/or purification as in 2) and 3) below, before separation by one of the procedures in section B.

Method b)

Method a) above was found to be inconvenient and time-consuming, so, as a routine method of extraction in later experiments, the leaf material was homogenised with the extraction solvents in a small Waring blendor. A sample extraction is given below.

20g of leaf material (fresh leaves stripped of their mid-ribs) was cut into approximately 2cm squares and placed into a small Waring blendor together with 0.1g of powdered calcium carbonate (this neutralised any acid cell sap released in the subsequent homogenisation). 40-60ml of acetone was added and the material was homogenised at full speed for one and a half minutes, then 30ml of n-hexane was added and the mixture rehomogenised for a further minute. The resultant mixture was filtered under suction and the residue, after washing with ether (freed of peroxide by standing over metallic sodium wire), was re-extracted in a similar manner. 100ml of saturated aqueous sodium chloride solution was carefully added to the combined extracts (plus ether washings) in a separating funnel. The lower, pale green aqueous phase was removed and washed twice with 20-25ml portions of peroxide-free ether and these washings were combined with the dark green organic layer from the initial separation. This total organic extract was then carefully

washed twice with distilled water, to remove the acetone, and dried over a little anhydrous sodium sulphate. The extract was decanted off and, for quantitative work, the sodium sulphate washed with a little peroxide-free ether. The combined extract plus ether washings were made up to a known volume, 200 or 250 ml, with peroxide-free ether and samples of this solution were taken for chlorophyll determination or chromatography (after partial purification, see 4), below) or evaporated down for lipid dry weight determinations.

Such extracts were normally taken to dryness in a rotary evaporator (bath temperatures of 45°C), but small volumes could conveniently be taken to dryness by placing them in a small Buchner flask, sealing with a rubber bung or ground glass stopper, connecting the side arm to a water pump (normally via a water trap in case of "sucking back") and evaporating off the solvent by gently swirling the flask in a water bath at about 45°C.

Large volumes could be extracted in a similar manner using a large Waring blendor as in the extraction of horse chestnut leaves for the isolation of plastoquinone-4 (Experimental Section E)

Method c)

Chayen, Smith, Tristram, Thirkell and Webb (1961) showed that milling leaf material in sodium hydroxide (by the Impulse Rendering process) liberated the constituents of the leaf yielding a dark green suspension of carbohydrates, lipids

and proteins. In an attempt to find out whether such an extraction had any effect on the pigments present a similar extraction procedure was adopted utilising a Waring blendor in place of the Impulse Rendering process. The mixture of carbohydrates, lipids and proteins produced after precipitation with acetic acid, was extracted with organic solvents to yield a similar lipid extract to those obtained by methods a) and b). This method of extraction (as method a)) was successfully used for both small (20g) and large (300g) batches of leaf material.

Experimentally, leaf material (leaves stripped of their mid-ribs) was homogenised for one and a half to two minutes in about five times its own weight of $^{N}/_{10}$ sodium hydroxide. The resultant dark green homogenate was filtered through cotton cloth (overnight at 4°C for large samples) and the residue rehomogenised in a similar volume of $^{N}/_{10}$ sodium hydroxide or distilled water. The homogenate produced was again filtered through cotton cloth and the combined filtrates were neutralised (to pH 4-5) by the careful addition, with stirring, of N acetic acid. The precipitate produced was centrifuged down at 2000r.p.m. for five minutes in a Martin Christ centrifuge, and the pale yellow-brown supernatant was discarded. The dark green precipitate was extracted by stirring vigourously with acetone or ethanol and the mixture again centrifuged. The supernatant was decanted off and the residue re-extracted several times

with various mixtures of n-hexane (or 60-80 petroleum ether) and either acetone or ethanol until no more green colour was extracted. The residue then consisted mainly of carbohydrates and proteins (equivalent to crude leaf protein).

The combined dark green extracts were washed with distilled water to remove the acetone or ethanol and the organic layer was dried over a little anhydrous sodium sulphate and evaporated down to dryness. The resultant residue was equivalent to that produced by methods a) and b) and its hexane solution was similarly subjected to chromatography, chlorophyll analysis and lipid dry weight determinations.

Some samples of the dark green mixture of carbohydrates, lipids and proteins, obtained by centrifugation of the initial suspension in sodium hydroxide after neutralisation with acetic acid, were taken and lyophilised in the laboratory freeze drier. The coarse, dark green powder obtained was stored at $-8^{\circ}C$ for two and a half months and then a sample was extracted by grinding with acetone and hexane. The extract obtained was then subjected to thin layer chromatography in an attempt to determine the effect of such treatment and storage on some of the carotenoids present.

3) SAPONIFICATION PROCEDURE

Many workers (e.g. Strain, 1938; Booth, 1957) have attempted to study the carotenoid pigments present in plant leaves using saponification to remove the chlorophylls and other interfering lipids (e.g. triglycerides) prior to

chromatographic or partition methods of separation. Quinones present in both animal and plant tissues are also frequently studied after saponification or alkaline extraction (Crane and Dilley, 1963). There is some disagreement as to whether or not saponification affects the carotenoids and quinones present, consequently some of the lipid extracts (produced by one of the methods in 2) above) were saponified or partially saponified in an attempt to examine this problem. Saponification at an elevated temperature can almost certainly be expected to result in some damage to both carotenoids and quinones and so only cold saponification was attempted in this work.

The major problem in obtaining a fairly rapid saponification was found to be the dissolving of the leaf lipid residue in a solvent which was miscible with the saponification medium. 10% potassium hydroxide in ethanol or N methanolic potassium hydroxide was used as the saponification medium in this work and it was found, as expected, that if the lipid residue was dissolved in ethanol and the alcoholic alakli was added, then saponification of the chlorophylls occurred fairly rapidly. Unfortunately, comparatively large volumes of alcohol were required to solubilise the lipid extracts and thus, during the subsequent separation, rather large volumes of water were required to transfer the saponified chlorophylls to the aqueous layer and leave the carotenoids (and quinones) in the organic (ethereal) phase.

It was found that the acetone/hexane mixtures dissolved the lipid residues fairly easily and only small volumes of such mixtures were necessary. Provided that the concentration of hexane in such mixtures was fairly low (less than 25%) then the resultant lipid solutions were completely miscible with the alcoholic alkali used for saponification. If the acetone-insoluble lipids were removed as in 4) below, then no hexane was necessary and the lipid residue was soluble in a small volume of acetone alone.

In a representative experiment the lipids obtained from 20g of leaf material (lipids extracted as in 2)b) above) were dissolved in 10ml of acetone in a 100ml conical flask (with the addition of 2-3ml of hexane if the acetone-insoluble material had not been removed as in 4) below) and 30ml of 10% ethanolic potassium hydroxide was added. The flask was stoppered, shaken and stood in the dark for 10-15 minutes with occasional shaking; in some experiments the air in the flask was displaced with nitrogen but in such a short time this did not appear to make a noticeable difference. The contents of the flask were now transferred to a separating funnel and 50ml of peroxide-free ether and 100-150ml of distilled water were added, in that order. The lower, aqueous green phase was extracted three times with 25ml portions of peroxide-free ether and the combined yellow organic phase and ether washings were washed three times with distilled water to remove alkali, acetone and ethanol.

The yellow ether solution was then dried over a little anhydrous sodium sulphate, concentrated under reduced pressure, and samples subjected to chromatography (see sectionB).

Some attempts were made to recover the saponified chlorophylls ("water soluble chlorophyll derivatives") from their aqueous solution by means of various adsorbents. For this purpose the aqueous green solution of saponified chlorophylls produced above was brought to pH6 with acetic acid. and the powdered adsorbent was added. The mixture was stirred and allowed to stand, then filtered. The adsorbents used were silicic acid (Mallinkrodt, 100 mesh), calcium phosphate and "low-temperature burnt magnesia" (a mixture of magnesium oxide and magnesium carbonate). These adsorbents were examined with regard to both the efficiency with which they removed the saponified chlorophylls from solution and the ease with which these derivatives could be recovered from the adsorbent by means of elution with acetone and methanol.

4) REMOVAL OF WAXES AND PHOSPHOLIPIDS

In order to estimate the amount of waxes and phospholipids present in the leaves examined, the lipid extract from about 50g of leaf material was saponified as in procedure 3) above and the yellow ether solution resulting was concentrated to 3ml. 15ml of AnalaR acetone was added to the ether solution and the mixture was left at

4°C overnight to precipitate the waxes and phospholipids; the plant waxes are said to be insoluble in 20-25% ether in acetone (Thirkell, 1961; Tristram, 1964). The flocculent waxy precipitate was removed and the solution again left overnight at 4°C, this procedure was repeated until no more wax was precipitated. The wax/phospholipid precipitation was also performed on extracts which had not been subjected to saponification, but the precipitate obtained was dark green and required several washings with cold acetone to remove the colour.

As a routine method of purification prior to chromatography, a sample of lipid extract (equivalent to 4-5g of leaf material) was taken to dryness in a small Buchner flask and the residue extracted three times with 2ml portions of cold, dry acetone. The acetone extracts were filtered through cotton wool or a small sintered glass funnel, combined and taken to dryness in another flask, then dissolved in a little ether or hexane for chromatography. Most of the waxes and phospholipids were left deposited on the walls of the flask in which the initial lipid extract had been taken to dryness. The filtration served to hold back any of this deposit that had been dislodged and this was washed back into the flask by the addition of a little hexane. The contents of this flask were then evaporated down and the weight of the dry residue was found.

For larger amounts of leaf lipid extract (equivalent

to 20g or more of leaf) the solution was taken to dryness in the presence of a small amount of washed Hyflo-Celite to provide a larger surface area for the acetone extraction.

This procedure was followed for both the saponified and unsaponified leaf extracts in later experiments, for the reasons discussed in the Results Section.

EXPERIMENTAL (B)

SEPARATION METHODS

1) COLUMN CHROMATOGRAPHY

a) Carotenoids

Column chromatography has been used classically by Strain (1938a, 1948b) and later by Moster, Quackenbush and Porter (1952), Bickoff, Livingston, Bailey and Thompson (1954) and others (see page 3) to separate the carotenoids of plant leaves. Initially, some experiments were performed in this work in an attempt to achieve a preliminary separation of the leaf pigments by column chromatography, prior to analysis of the fractions so obtained by TLC. In actual fact, the carotenoid separations obtained by this column chromatography were not very satisfactory and so no further analysis was performed on the fractions eluted from the column. The main adsorbent used for column chromatography in this work was silicic acid (Malinkrodt, 100 mesh) and columns were prepared either by dry packing of the adsorbent or by packing it in a slurry in n-hexane.

b) Quinones

Column chromatography was also tried for the separation of the isoprenoid quinones, partially in view of their instability to oxygen and light (which became apparent during the course of these investigations) which may render other forms of chromatography unreliable, and partially in order to examine larger amounts of leaf extract. Various

adsorbents were tried including magnesia/hyflo and silicic acid/hyflo mixtures in various proportions and also silicic acid and alumina. The best initial separations appeared to be on alumina and in view of the reported effects of alumina on the isoprenoid quinones (Hemming, Morton and Pennock, 1961) a deactivated grade was used. In early experiments Spence Grade "O" alumina (Brockmann Grade II-III) was used, but then Woelm neutral alumina, deactivated with water to Brockmann Grade III, was found to give more reproducible results and so was used in all later work.

The LKB Uvicord was used to monitor the column effluent at 254nm and consequently the solvents used had to be fairly transparent to UV light of this wavelength. Various non-polar hydrocarbon solvents (n-hexane, cyclohexane, heptane) were available and 1,2-dichloroethane was used as the polar eluting solvent.

In early experiments the alumina was packed, as a slurry in n-hexane, in a 30 x 1cm glass column fitted with a sintered glass disc. The 20g of alumina normally used however, only formed a packing of about 23cm high in such a column and so a dead-space was left at the top. This dead-space filled with solvent during the course of a chromatographic run and could be expected to alter the applied gradient, consequently a column head was devised which could leave little or no dead space. Initially this took the form of a plug made out of teflon tubing and teflon

tape, which could be pushed tightly into the bore of the column. Later the device shown in fig. 28 was designed and this was made in the department workshop. The teflon ring was cut from a piece of wide bore teflon tubing and could be compressed against the walls of the column by means of the nut and screw thread on the inner copper tube.

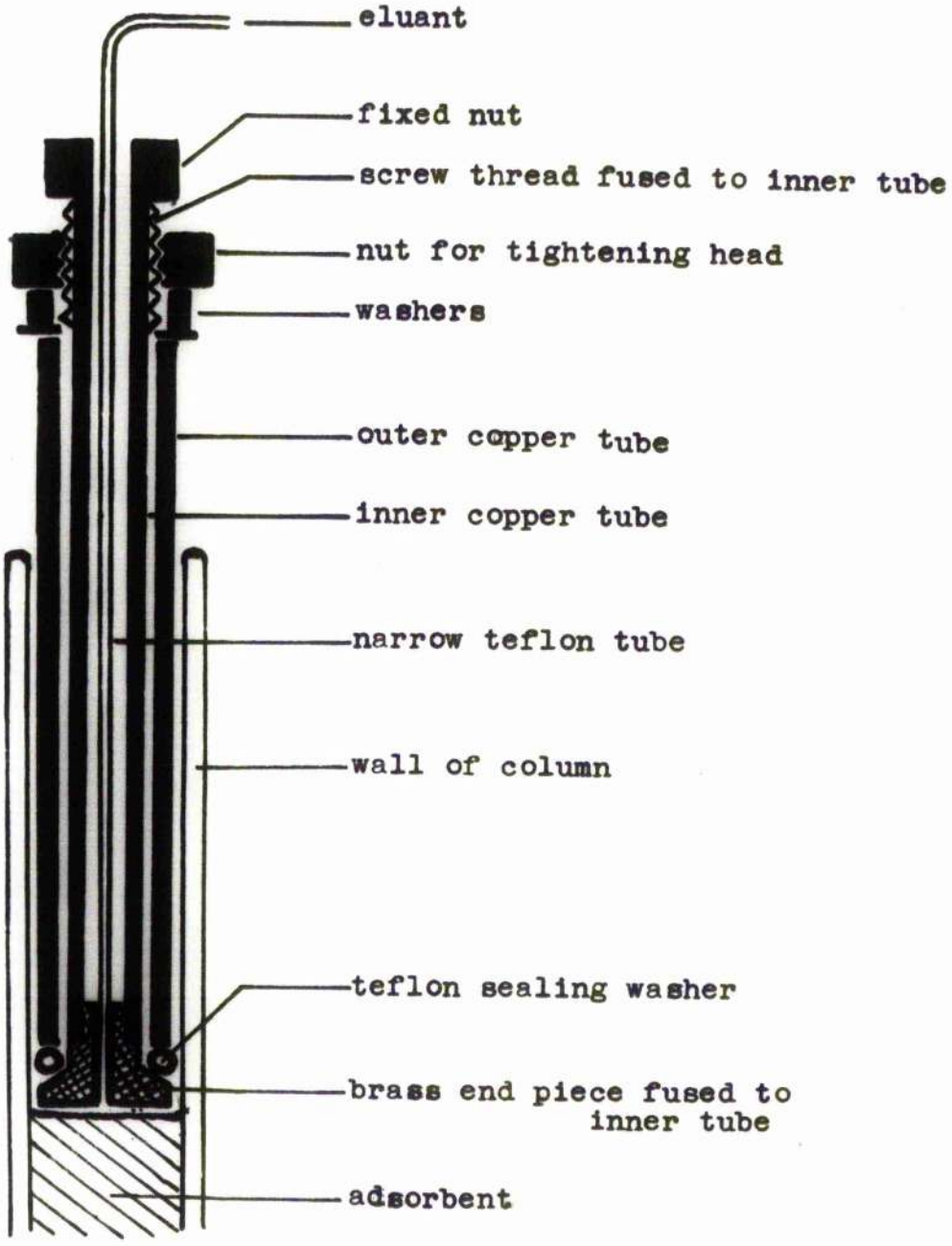
The adaptor shown allowed different length column packings to be set up in the same column with no dead space and separations were attempted with 5g, 10g, 15g, and 20g of columns of Alumina.

Rapid separations were required in this work and Vestergaard and Sayegh (1966) and Vestergaard, Sayegh and Witherell (1966) report the rapid separation of steroids using capillary columns. In view of the results of these workers some experiments were carried out to see if rapid separations of the quinones could be achieved by using narrow columns. Initially narrow glass columns (inside diameter of 0.6cm) were used with a length of either 85cm or 200cm (U-shaped) but these were somewhat fragile and columns made of teflon tubing (0.5cm inside diameter) were later used. For the reasons discussed in the Results Section, 100cm was the final length of column used.

For the connections to the columns, fine bore teflon tubing was used throughout and connections were made using "sleeving" teflon of a tight fit. Where connections were under pressure the sleeving teflon was replaced by viton and wired to ensure a tight fit. The connections at the

Fig. 28

Column-head design



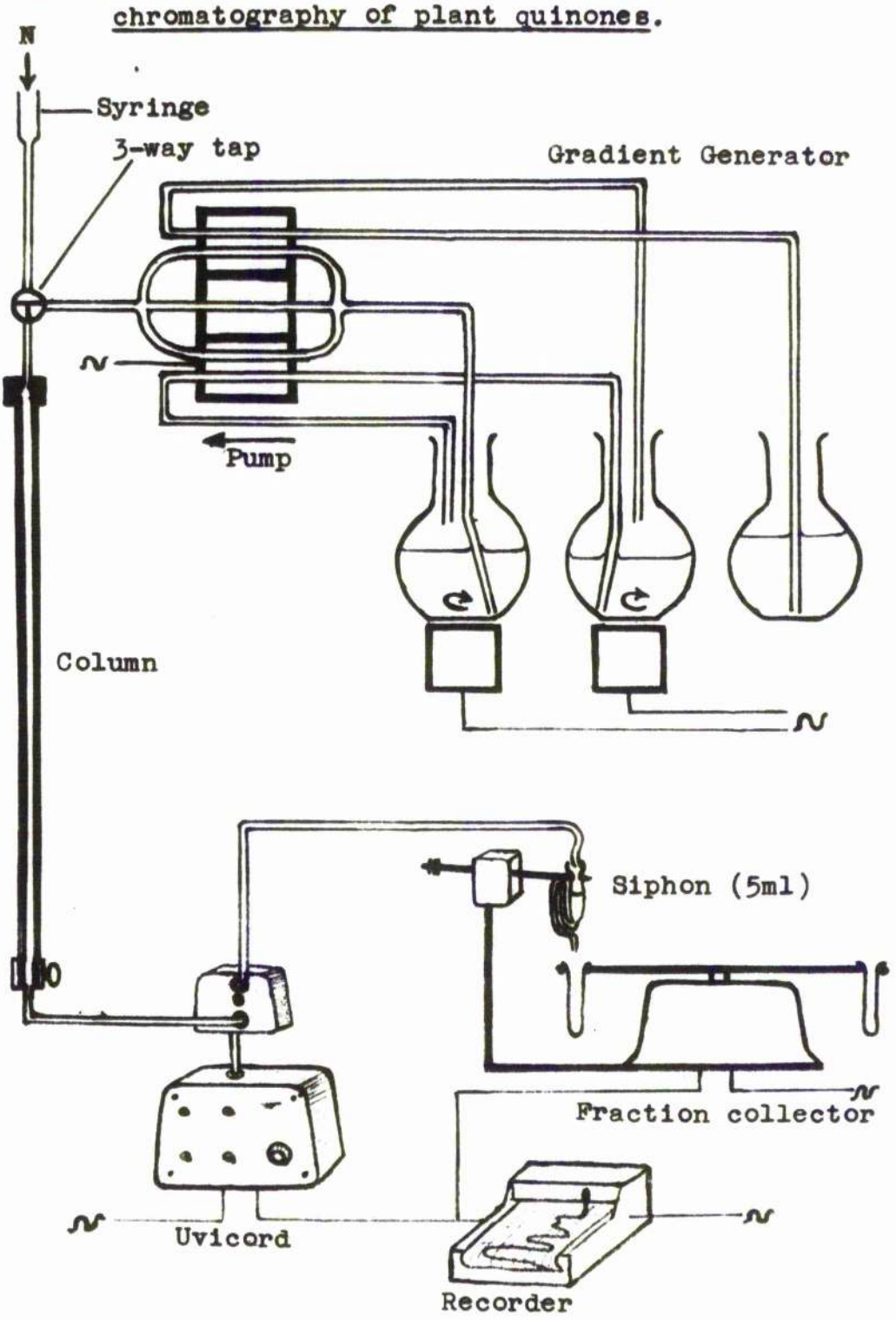
column inlet and outlet were made with teflon and viton "plugs" in the case of glass columns. With the teflon columns the inlet connection was made by means of a "swagelok" fitting and the outlet by means of a teflon plug and a circular clamp. This teflon plug supported a small piece of washed cotton wool which held back the adsorbent packing.

The elution gradients were produced by a system of pumps as described in the Gradient Elution Section and a three-chamber device was found satisfactory for this work. Initially the pumps used were DCL Micro pumps, but in later work these were replaced (for reasons discussed in the Results Section) by a Durrum twelve channel "positive displacement tubing pump" fitted with solvent-resistant viton tubing. To minimise "pulsing" of the flow in this system, three tubes were connected in parallel but out of phase with one another to supply the column. Single channels only were used to supply the mixing chambers.

As was stated earlier, an LKB Uvicord flow analyser was used to monitor the optical density of the column effluent at 254nm and a diagram of the final arrangement used for the phytoquinone separations is shown in fig. 29. For quantitative work, the normal Uvicord recorder was replaced by a Vitatron potentiometric recorder which had the advantage of a larger chart area and variable speeds. The recorder chart was normally operated at a speed of 15cm per hour and 5ml fractions, collected by siphon from the column,

Fig. 29

Column assembly for the gradient elution chromatography of plant quinones.



were marked by means of the event marker in the LKB fraction collector used. This device made a temporary short circuit in the input signal, thus causing a vertical line in the recorder trace. This fraction marking was useful for locating the collected fractions, but was also found to be necessary for determining peak volumes, as the flow rate tended to change with the varying solvent gradient (see the Results Section).

The fractions eluted from the column were characterised by TLC, paper chromatography and spectrophotometry as described in later parts of the Experimental Section. For quantitative work the eluted quinone fractions were determined by the borohydride reduction assay (q.v.), but for routine measurements the peak area on the recorder trace was measured. This area was determined by finding the optical density of the peak maximum and halving this after correction for any base-line deviation. The volume at this half peak height could then be found from the trace and the peak area was calculated by multiplying this volume by the corrected peak height. The area thus calculated was in units of volume times optical density (ml.OD units) and the mass of substance was then determined either by comparison with known standards (in the case of phylloquinone, ubiquinone and plastoquinone A) or from a consideration of the extinction value ($E^{1\%}_{1cm}$) of the compound and the path length of the flow cell used (0.75cm). These extinction values were calculated

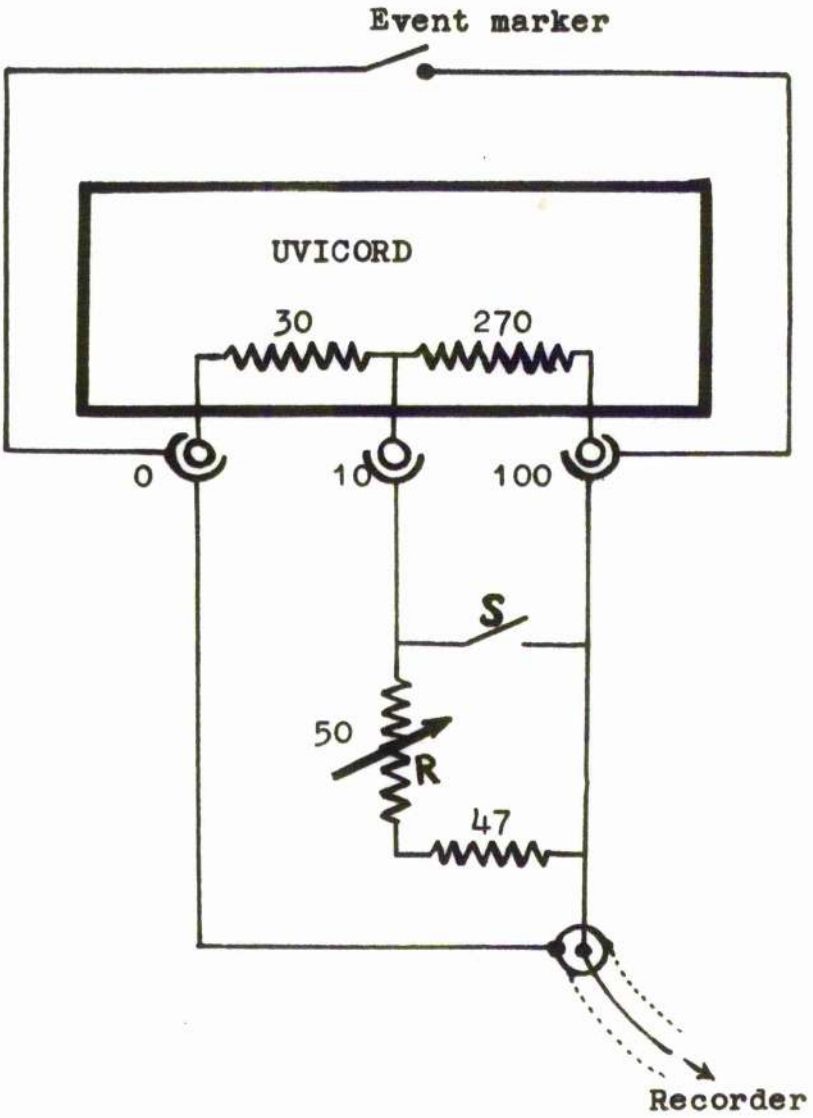
from the molecular weights of the plastoquinones according to Das, Lounasama, Tendille and Lederer (1965, 1967) and from the extinction value of 253 for plastoquinone A given by Isler, Rüegg, Langemann, Schudel, Ryser and Würsch (1961). The extinction value for plastoquinone B was calculated as 190 and for the plastoquinone C and D group as 249.

Since satisfactory separations of α-tocopherol quinone and some of the plastoquinone C and D group were not obtained due to the presence of steroid (see the Results Section) the fraction containing these compounds was collected and assayed by the method of Bucke and Hallaway (1966); this is described in Experimental Section C on spectrophotometry.

Although the Vitatron recorder was normally operated in a linear fashion (i.e. measuring transmittance) it was sometimes necessary to operate it as a log recorder as in the measurement of dichromate concentration in the Gradient Elution Chromatography Section. It was felt preferable to be able to change directly from the linear to the log scale of the Vitatron, but unfortunately these two scales are not equivalent: the linear scale requires 10mv for full scale deflection, whilst the log scale requires 30mv. In order to overcome this difficulty the circuit shown in fig. 30 (designed by Dr. Stevens) was used to modify the input signal to the recorder. The switch S is closed for linear (10mv) operation and left open for log (30mv) operation. The two scales can be made directly equivalent by adjusting

Fig. 30

Modifying circuit for Vitatron recorder



the variable resistance R so that the same signal gives a reading of 1.0 on the log scale and 10% on the transmission scale, whilst zero on the log scale is equivalent to 100% on the transmission scale.

c) Routine separation of the phytoquinones

For routine determinations a 100 x 0.5cm column of teflon tubing was set up with a teflon and cotton wool plug at its lower end; the fine teflon tube from this plug was closed by means of a short length of clamped viton tubing. A small funnel was attached to the top of the column and both the column and the lower half of the funnel were filled with n-hexane. 20g of Woelm Alumina (Grade III) were now poured into the funnel and allowed to settle into the column. When a depth of about 5cm of alumina was visible at the base of the column the outlet was opened to allow the adsorbent to settle more rapidly; the settling was also assisted by occasionally tapping the column with a spatula. When all the alumina had settled it was compacted by pumping a small volume of n-hexane through the column and more alumina (1-2g) was then added to fill the space so made. The column was then preconditioned by pumping 25ml of 50% dichloroethane/ n-hexane through it, followed by 25ml of n-hexane. This procedure was adopted in order to prevent any discontinuous concentration change as suggested by Wren (1963). A known weight of leaf (10-25g) was then taken and extracted as in Experimental Section A 2)b). The hexane extract was made

up to a known volume and a sample equivalent to 0.5g taken for chlorophyll determination as in Experimental Section (C). The remainder of the leaf extract was washed once with its own volume of 80% aqueous methanol and then once with an equal volume of 90% aqueous methanol, to remove the polar lipids. The extract was taken to dryness and acetone extracted (Experimental Section A 4)) to remove the waxes present. The acetone extract was taken to dryness, dissolved in 1-2ml warm n-hexane and transferred to the syringe body shown in fig. 29. Nitrogen pressure of 8-10lbs/sq. inch was then applied to the syringe and the extract was forced onto the column via the three-way tap. The flask which had contained the extract was rinsed twice with 1-2ml n-hexane and these washings were similarly loaded onto the column. The three-way tap was then turned so as to connect the pump to the column and a gradient of dichloroethane in n-hexane was applied to separate the quinones. The initial chamber conditions were (0.75/150/100)(1.5/150/2.4)(2.5/250/2.4) and the gradient produced by this "imperfect" system is shown together with the separations achieved, in the Results Section.

The separation was allowed to run for three hours, and the fraction eluted after about two and a quarter hours was collected and assayed for α-tocopherolquinone and plastoquinone D as described in Experimental Section C 3).

EXPERIMENTAL (B)

2) THIN LAYER CHROMATOGRAPHY

a) General Principles

Thin layer chromatography (TLC) appears to have been first used by Izmailov and Shraiber (1938). The technique was revived by Kirchner and Keller (1950) and Kirchner, Miller and Keller (1951) but it was not until the systematic work of Professor Stahl (1956, 1965) that the wide range of application of TLC could be appreciated. Stahl simplified and, to a great extent, standardised the techniques of TLC, particularly with regard to the preparation of uniform thin layers of adsorbent. Stahl's original work led to the designing of an apparatus for the application of uniform adsorbent layers to several glass plates at once and such an apparatus, manufactured by Messrs. Desaga, of Heidelberg (and obtained through Camlab Ltd., of Cambridge), was used in some of this work. An alternative applicator, marketed by the Shandon Scientific Company Ltd., was also used and slightly more even layers were obtained with this type of apparatus although it was found difficult to standardise the thickness very accurately. The Quickfit and Quartz TLC applicator used for most of this work was simpler to use than the Shandon apparatus and gave equivalent layers; it could produce adsorbent layers of 250μ, 500μ, 750μ, or 1000μ (as laid). The Desaga spreader used gave a standard

film thickness of 250µ, whilst the Shandon "Unoplan" was adjustable for adsorbent layers of thicknesses between about 200 and 2000µ.

Basically an applicator for TLC is required to produce an even, reproducible layer of a slurry of the adsorbent (usually in water) to one or more plane glass plates. The glass plates used in this work were 20 x 20cm, 20 x 10cm and 20 x 5cm. The adsorbent mainly used in TLC today (and that used by Stahl) is Kieselgel G (or Silica Gel G) which is a silica gel of fine particle size, containing about 10% of gypsum (calcium sulphate) as a binder to help the layers adhere to the glass plates. In much of this work 250µ or 500µ layers of Silica Gel G or Silica Gel H (q.v.) were used and these were dried at room temperature for 10-20 minutes (longer for binder-free adsorbents) and then activated by heating before use (see b) below). Layers of Aluminium Oxide G were treated similarly.

The TLC plate holding the adsorbent layer was loaded (as is general in TLC) with the mixture to be chromatographed (leaf extract in this work) by application as a spot about 2cm from the lower margin of the plate (and at least 1cm from the edge to prevent "edge effects"). A 10µl pipette was used for such dropwise applications. For preparative work it was necessary to apply the leaf extract as a strip or band along the origin and this was best accomplished by means of a 250 or 500µl pipette with a small piece of teflon

tubing fitted over the end of the stem, so that the pipette could be drawn along the perspex template provided (with the tubing resting on the template) without actually touching the adsorbent layer. The drop at the end of the pipette touched the adsorbent and served to transfer the leaf extract to the plate by capillary action. With practice, excellent results could be obtained using this method of strip loading. The strip (or spot) was then dried off with an air blower before development of the chromatogram.

The Desaga preparation box (obtained from Camlab Ltd.) was also used particularly for preparative TLC of the isoprenoid quinones. Strip loading under nitrogen could be accomplished by means of a micropipette fitted with a collar of teflon tubing as above. This collar was then allowed to rest on the edge of the loading orifice and the flow rate was controlled by means of a finger on the top of the pipette; the slide could then be moved along evenly with the other hand.

The TLC plates were then developed by standing them almost vertically in about 1cm depth of solvent (see c) below) in a small chromatography tank with a well fitting lid. The tank was covered with a light-proof cloth to prevent photochemical destruction of any of the compounds present. Development normally required 30-50 minutes, and in this time the solvent usually rose to a height of about 10-15cm. After this time the plates were removed

from the tank and the solvent front was marked. The chromatograms were then examined in visible and ultra-violet light and any components thus detected were marked with a sharp pencil. The pigments were easily visible as spots or bands, but for the detection of colourless compounds such as quinones and tocopherols the TLC plates were sprayed, after development, with one of the detection reagents described in d) below.

For examination of specific lipids the bands of adsorbent holding them were scraped from the plate with the aid of a spatula and the lipids were eluted with ether or ethanol, and these extracts filtered or centrifuged to remove the adsorbent. In the case of the colourless quinones a small strip of the plate about 2cm wide was sprayed with a detection reagent, the remaining area being covered with a glass plate. The strips of adsorbent on the unsprayed portion of the plate, which corresponded to the coloured bands of the sprayed portion, were removed and eluted as above. The extracts obtained were normally examined spectrophotometrically (see Section C).

Some experiments were also performed using a "saturated atmosphere" in the developing tank. This was obtained by lining the walls of the tank with Wahtman 3MM paper saturated with the solvent. The "S-chamber" technique of Stahl (1965) was also used in much of this work and good results were obtained with the Desaga apparatus. In this

technique the TLC plate itself forms one wall of the tank and a cover plate, with 0.3cm glass strips sintered along three edges, is clipped on to formthe other walls. With this technique, saturation of the chamber is very rapid and only small volumes of solvent are required to fill the developing trough into which the assembled chamber is placed. Davies (1963) has described a simplified form of the S-chamber although in this work the sintered glass cover plate was found to be superior. Adsorbents and solvent systems used are described in b) and c) following, and their merits are discussed in the Results Section.

Preservation of thin layer chromatograms (where these had not been used for preparative separations) was achieved by spraying the adsorbent layer with "Neatan" (Merck). This is an aqueous dispersion which impregnates the adsorbent film to seal it in a thin plastic sheet. When dry, the complete adsorbent film was removed from the glass plate by placing it in a dish of water and gently prying off the layer with a spatula. The film was then dried, stuck onto sheets of paper with adhesive tape and filed.

b) Adsorbents used

Although Stahl originally used Silica Gel G, other adsorbents are now available for TLC and some of these were experimented with. The actual adsorbent, the way it was used to prepare plates and the treatment of the plates prior to loading and development, are described below. The separations obtained on such plates with various solvents are discussed in the Results Section.

(i) Silica Gel G (Merck): This adsorbent was used in most of the work. To coat five 20 x 20cm plates with a layer 250μ thick (as spread), 30g of this adsorbent was mixed vigourously with 40ml of distilled water until no lumps remained. A further 20ml of distilled water was then added and the slurry was again stirred vigourously and poured into the applicator. The whole process of mixing the slurry and coating the plates was carried out fairly rapidly, as the gypsum present caused the mixture to set within four or five minutes. The plates were dried for 10-20 minutes at room temperature and then activated by heating at 110°C, for at least an hour, before use.

(ii) Silica Gel G (Macherey): Almost identical to the Merck product and was treated similarly. A sample of the adsorbent was also purified, by extraction with hexane/ethanol (50:50) and then ethanol. Plates were prepared from this purified material in an identical manner.

(iii) Silica Gel G/UV254 (Macherey): A similar product to the silica gel above but containing an inorganic fluorescent indicator to aid in the detection of compounds which absorb ultra violet light. The layer appears a brilliant yellow-green under ultra violet light, whilst absorbent compounds show as a dark grey. Plates were prepared similarly to the other silica gels above.

(iv) Silica Gel H (Merck): A binder-free silica gel of fairly high purity which was used a great deal, especially

for the chromatography of the quinones. Both 250μ and 500μ layers were used and to coat five plates 20 x 20cm with a layer of 250μ, 30g of the adsorbent were shaken in a conical flask with 70ml of distilled water. As no binder was present the slurry could be left to stand before use. The plates were allowed to dry at room temperature for about an hour and were then activated for 1-2 hours at 110°C.

(v) Silica Gel (Woelm): A binder-free silica gel of fairly high purity which was used for the preparation of adsorbent layers 500-600μ thick (as spread). To coat five plates 20 x 20cm with a layer 500-600μ thick, 70g of the adsorbent was mixed by hand with 95ml of distilled water. As no binder was present the slurry could be left to stand before use. The plates were dried and activated similarly to those of Silica Gel H above.

(vi) Silica Gel SG41 (Whatman): A binder-free silica gel similar to the Woelm product above although slightly different conditions were necessary to produce comparable layers. To coat five plates 20 x 20cm with a layer 600μ thick, 50g of the adsorbent was homogenised in 100ml of distilled water in a Waring blendor. The plates were dried and activated similarly to those prepared from the Merck and Woelm products above.

(vii) Aluminium Oxide G (Merck): This adsorbent was used for a number of separations of the quinones, and plates were prepared in a similar manner to those of Silica Gel G

(viii) Mixed adsorbents

Various mixed adsorbents were tried including magnesia/hyflo mixtures, calcium carbonate/hyflo mixtures and aluminium oxide/cellulose mixtures, but none of these gave very satisfactory results. Good results were obtained however with a 50/50 (w/w) mixture of Aluminium Oxide G and Silica Gel G which Stahl (1965) describes as "Alusil". These plates were prepared similarly to those of Silica Gel G above.

c) Solvents used

A large number of different solvent systems were tried during this work for the separation of the carotenoids and the quinones present in leaf extracts. The best of these solvents are listed below with respect to the compounds which they satisfactorily separated and the adsorbents on which they were used. Since silica gel (with or without gypsum binder) and alumina (with gypsum binder) were the major adsorbents used these are the only ones considered below.

(i) Separations of the carotenoids on silica gel

Satisfactory results were achieved with the following solvents.

Benzene/acetone (60:40)

Hexane/acetone (50:50) (60:40)

Hexane/ether (70:30)

The best of these solvents was found to be hexane/

acetone (60:40) using AnalaR acetone and an unlined developing tank. Some of the less polar carotenoids were better separated by means of some of the solvents in (ii) below.

(ii) Separations of the quinones on silica gel

Satisfactory results were achieved with a number of solvent systems including the following.

Benzene

Benzene/acetone (98:2)

Benzene/ethanol (98:2)

Benzene/hexane (varying proportions)

Carbon tetrachloride/acetone (95:5)

Chloroform

Dichloroethane

Dichloroethane/hexane } (varying proportions)
Dichloroethane/heptane }

Good results were obtained with benzene and benzene/hexane mixtures both in lined and unlined tanks as well as in the S-chamber. Dichloroethane and dichloroethane/hexane mixtures were useful for the separation of the more polar quinones under the same conditions.

(iii) Separations of the quinones on aluminium oxide (alumina)

Alumina plates were useful for some separations which were relatively unsatisfactory on silica gel (see the Results Section) and similar solvent systems were used. Best results were obtained with the following, both on alumina and alusil plate.

Benzene

Benzene/hexane (varying proportions)

Dichloroethane/cyclohexane }
Dichloroethane/heptane } (varying proportions)
Dichloroethane/hexane }

Toluene

d) Detection Reagents

(i) The "Phase-Test" for chlorophylls: This test is based on the reaction of chlorophylls with 10% potassium hydroxide/methanol and consequently the plate was sprayed with this solution and the colour changes noted (see Smith and Benitez, 1955, pp 192-193).

(ii) The "Carr-Price" test: This relies on the reaction of carotenoids and vitamin A with antimony trichloride/chloroform to give a blue colour. The spray reagent was prepared by carefully grinding antimony trichloride with chloroform, in a mortar and pestle, to give a saturated solution. The whitish suspension was allowed to settle and then the TLC plate was sprayed with the fresh solution (not more than six days old) and the colours noted. On heating the plate, steroids became visible as pinkish spots (Merck, 1963).

(iii) Rhodanine: This reagent was used as a 1% solution in ethanol. Weakly coloured carotenoid aldehydes give a red colouration with this reagent in the cold on overspraying with concentrated ammonia (Truter, 1963, p47).

(iv) Liebermann-Burchard test: A modification of this test for steroids was performed by spraying the TLC plate with a mixture of acetic anhydride and chloroform (1:1) and then overlaying the surface with a glass plate smeared with concentrated sulphuric acid. Steroids reacted rapidly in the cold to give characteristic blue-green colours (other transitory colours were also observed).

(v) Neotetrazolium chloride: Many experiments were performed to find a modification of this reagent of Lester and Ramasarma (1959) for the detection of quinones on paper chromatograms, which would be suitable for use with TLC. Initially fairly good results were obtained by spraying the TLC plate first with a 0.1% solution of potassium borohydride in 50% aqueous ethanol followed, after 20-30 seconds, with a 20% solution of concentrated hydrochloric acid in ethanol to destroy the excess borohydride. Spraying with a 0.25% solution of neotetrazolium chloride in 50% aqueous ethanol and then warming with a hot air blower revealed the quinones as purplish pink spots. This procedure was somewhat cumbersome and better results were later achieved by reversing the order of reagents and omitting the acid. The final form in which this reagent was used was by spraying the TLC plate with 0.25% neotetrazolium chloride in phosphate buffer (pH 7.0), drying off with an air blower and then overspraying carefully with 0.1 - 0.5% aqueous potassium borohydride. Warming gently from below

with a hot air blower revealed the quinones, as above, as purplish pink spots. Where the reagent was used on silica gel plates the phosphate buffer could be replaced by ethanol but on alumina or alusil plates this caused the whole adsorbent layer to turn pink (probably a pH effect).

Under the conditions described, this reagent was found to be very useful for the detection of quinones present in the lipid extracts of leaves.

(vi) Triphenyltetrazolium chloride: Attempts were made to use this reagent as a less expensive substitute for neotetrazolium chloride in the procedure above, but although some results were obtained, sensitivity of the test was not nearly as great. The reagent was also used as a 4% solution in 5% alcoholic alkali; under these conditions it should produce a red colouration with reducing compounds (Merck, 1963) but there was a tendency for the whole adsorbent layer to turn red.

(vii) Leucomethylene blue: Two forms of this reagent for quinones were tried. The first was prepared according to Goodwin (1964) by adding 1ml of glacial acetic acid and 250mg of zinc dust to 5ml of a 0.02% solution of methylene blue in acetone. The mixture was swirled gently until almost colourless and then the TLC plate was sprayed with this solution.

The second form of reagent was prepared according to the method of Linn, Page, Wong, Gale, Shunk and Folkers (1959)

by adding 1gm of zinc dust and 1ml of glacial acid to 100mgm of methylene blue in 100ml of ethanol. Only this latter form of reagent seemed to give satisfactory results on the TLC plate, the quinones present giving a blue colouration almost immediately (the whole plate turns blue in about five minutes).

(viii) Ferric chloride/$\alpha\alpha'$-dipyridyl reagent: The spray reagent was prepared by mixing one part of 0.5% ethanolic ferric chloride with one part of 1% ethanolic α,α'-dipyridyl. This reagent was used for the detection of tocopherols and also of the hydroquinones produced by reduction of the quinones present with potassium borohydride as in the first method in (v) above.

(ix) Turnbull's Blue reagent; (Skinner, Parkhurst and Alaupovic, 1964). The TLC plate was sprayed with a 5% aqueous solution of ferric chloride, allowed to dry slightly and then oversprayed with a 5% aqueous solution of potassium ferricyanide. Tocopherols present reduced the ferric chloride to ferrous which gives a dark blue colouration (Turnbull's Blue) with the potassium ferricyanide. This was found to be a very sensitive test for tocopherols.

(x) Phosphomolybdic acid: This reagent was used according to Seher (1961) as a 20% solution in ethanol to test for antioxidants. After spraying the TLC plate with the solution, tocopherols (and other antioxidants if present) gave a dark blue colouration. The plate was left for

about three minutes and then sprayed with concentrated aqueous ammonia which rendered the yellow background colourless, thus making it easier to distinguish any faint blue colouration.

(xi) Diazotised o-dianisidine (Diazo reagent): (Analytical Methods Committee, 1959). A freshly prepared solution of 0.5% aqueous Fast Blue Salt B (Merck) was sprayed onto the plate and allowed to dry slightly. The plate was then oversprayed with 0.1 N sodium hydroxide to speed up the colour development. This reagent was used to distinguish between α- and β-tocopherolswhich gave a faint pale brown colouration and γ- and δ-tocopherols which reacted to give a dark purplish colouration.

(xii) Fluorescein: This reagent was used both as its potassium salt (0.1%) in aqueous solution and as a 0.01% solution in ethanol (Dunphy, Whittle and Pennock, 1965) to detect ultraviolet absorbing compounds. Several non-absorbing compounds are visible on damp plates since they react with the yellow fluorescein to yield eosin which is red. Observation under ultra violet light (254nm) shows absorbing and other reactive compounds as dark areas on a brilliant yellow-green fluorescent ground.

(xiii) Rhodamine B: This reagent was used as a 0.5% solution in ethanol (Merck, 1963) as a general spray reagent for lipids and for the detection of ultra violet absorbing compounds. Better results were obtained with a 0.01% solution as used

by Beiss (1964).

(xiv) Rhodamine 6G: This reagent was used in a similar manner to Rhodamine B above. Better results were obtained by preparing the TLC plates with 0.1% aqueous Rhodamine 6G in place of distilled water, as described by Avigan, Goodman and Steinberg (1963). When viewed under ultraviolet light (254nm), absorbing compounds (quinones and tocopherols) were visible as dark areas on a yellow fluorescent ground; neutral lipids such as steroids were visible by their enhanced fluorescence.

(xv) Sulphuric acid: This was used as a general detection reagent for organic compounds. Good results were obtained by spraying the plate with conc. H_2SO_4/ethanol (1:2) and then heating at 110° for ten minutes. All organic compounds were revealed as dark brown or black areas under these conditions and in the early stages of the reaction any steroids present gave characteristic blue, green or red colourations.

(xvi) Iodine: Iodine vapour was used as a general lipid detection reagent by either blowing the vapour from a warmed flask onto the plate, or by allowing it to stand in a TLC tank containing a few crystals of iodine. Any lipids present were revealed as yellow or brown spots with these techniques.

(xvii) 1-methylquinaldinium methosulphate: Sawicki, Stanley and Hauser (1959) have described the use of quinaldinium

and lepidinium salts in alkaline solution for the colorimetric detection of terminal ring quinones in air pollution studies. Attempts have been made in this work to use this reaction for the detection of the biological quinones, with some success as reported in the Results Section.

The 1-methylquinaldinium methosulphate was prepared by slowly adding 6ml of quinaldine to 4ml of dimethyl sulphate in an ice bath. This mixture was then allowed to stand for 30 minutes at room temperature when it solidified. The solid product was recrystallised from 100ml of n-propanol by cooling the hot solution in an ice bath. The off white crystals obtained were rinsed with a little n-propanol air dried and then stored at 0°C. The quinaldinium salt was used as 2.5% solution in methoxyethanol.

EXPERIMENTAL (B)

3) MODIFICATIONS OF THIN LAYER CHROMATOGRAPHY

Several modifications of TLC were tried in this work and these are described below. They can be divided into two types, the first or which utilised the "reversed-phase" technique either wholly or partly (a) and b) below). The other type of modification (c) - e) below) effectively extends the length of the solvent run, with either a single solvent increasing the size of the plate, or with different solvents.

a) Reversed-phase partition TLC

Hydrophobic TLC layers can be used with polar solvent mixtures to achieve lipid separations by partition instead of adsorption chromatography. Since different factors are operating it is usually possible to separate individual lipids on reversed-phase plates even when they have very similar Rf values on adsorbent layers. A number of different hydrophobic layers were tried in this work and these are described below.

(i) Paraffin-impregnated silica gel

Goodwin (1964) has described the use of silica gel layers impregnated with liquid paraffin to separate the ubiquinones and such layers have been used in this work. The plates were prepared by placing normal silica gel plates into a TLC tank containing 200ml of 5% (v/v) liquid paraffin

in n-hexane and the tank was then closed and slowly tilted to immerse the whole plate. After 30 seconds the tank was slowly tilted back to the vertical, opened and the plate removed. After drying in the air for one minute the reversed phase plate was ready for use. The solvents used on such layers included 2-10% water/acetone and 98% absolute ethanol, all previously saturated with liquid paraffin.

(ii) Polyamide layers

Layers of polyamide and polyamide/cellulose mixtures have been used by Egger and Kleinig (1965) for the separation of lipid soluble quinones and similar layers have been used in this work. Egger and Kleinig used cellulose to stabilise their polyamide ("Perlon") layers but the Macherey, Nagel product used in this work gave satisfactory layers without any binding or stabilising agents. Solvents of 0-20% water in acetone were mainly used on these layers.

(iii) Polyethylene powder layers

Since polyethylene should produce a hydrophobic layer, attempts were made in this work to produce satisfactory layers of this material. Good results were achieved with the product by passing I.C.I. Alkathene powder or BDH polyethylene powder through a 100 mesh sieve. 12g of this fine powder was homogenised for one minute in 50ml of methanol and used to coat 5 20 x 20cm plates with a 250µ layer. Similar solvents were used as on the polyamide layers above.

(iv) Silicone rubber

(iv) Siliconised cellulose

Only a column grade of this material was available but coarse layers could be prepared by hand by rolling out a slurry with a glass rod. Some separations of the quinones were obtained on these layers using methanol as a solvent. Reversil-3 (Applied Science Laboratories Inc.), which is a silica gel treated with dimethyldichlorosilane, should give superior results.

b) Two dimensional TLC using adsorbent and reversed-phase techniques.

Two procedures have been tried in order to achieve both normal and reversed-phase chromatography on a single TLC plate; these are described below.

(i) Silica gel/paraffin impregnated silica gel

In this method a normal silica gel layer was spotted at one corner with the mixture to be separated (usually either a crude or partially purified leaf extract). The plate was then developed in the normal manner in a solvent such as benzene and, when development was complete, removed from the tank and allowed to dry. The unoccupied part of the plate was then impregnated with liquid paraffin, dried and developed at right angles to the initial direction in a reversed-phase solvent such as 5% water/acetone. Some attempts were made to impregnate the layer by spraying it with liquid paraffin in either n-hexane and better results might be obtained by this technique if an even spraying could be achieved.

(ii) Silica gel/polyamide or polyethylene powder

A novel method was devised whereby TLC plates could be prepared with a narrow strip of silica gel for the first development and a large area of hydrophobic layer for the second. To prepare such plates a normal (Quickfit) spreader was modified by inserting a plastic division 4cm from one end. A silica gel slurry was then poured into the narrow section formed and a polyamide or polyethylene slurry into the remainder; plates were then laid as usual. It was found advantageous to slurry both materials in the same medium to produce an even junction and 50% aqueous methanol was found to be best for this purpose. Good separations were achieved on these plates and these are described in the Results Section.

c) Two dimensional adsorbent TLC

Various attempts at two dimensional TLC were made, using Silica Gel G with hexane/acetone (60:40) as the first solvent. After the solvent front had risen to a height of about 12-15cm the plate was removed from the tank and dried off with a cold air blower or a jet of nitrogen. The plate was then turned through 90° and developed in the second solvent, again to a height of 12-15cm (or more if necessary). The solvents used in the second direction were some of the less polar solvents listed in 2)c) above, e.g. benzene and benzene/ethanol (98:2).

d) Multiple development

This modification of TLC was tried, particularly for the separation of some of the quinones present, by developing the chromatogram to a height of only 6-10cm in the first solvent, removing the plate from the chromatography tank and drying off the solvent as in a) above. The TLC plate was then redeveloped in the same direction to a height of 12-15cm with the second, less polar solvent, thus giving a better separation of compounds running with the solvent front in the first solvent. Pairs of some of the less polar solvents listed in 2)c) were tried, particularly benzene/acetone (98:2) or benzene/ethanol (98:2) followed by benzene.

e) Continuous TLC

Continuous TLC has been performed using the BN chamber as described by Stahl (1965); the apparatus used was manufactured by Desaga and obtained through Camlab Ltd. Cyclohexane and heptane/dichloroethane mixtures were used to separate phylloquinone and plastoquinone A on layers of alumina and development times of up to 8 hours have been used. Continuous development of silica gel plates with 40-60 petroleum ether (AnalaR) was used to separate α- and β-carotenes in leaf extracts.

EXPERIMENTAL (B)

4) PAPER CHROMATOGRAPHY

Ordinary paper chromatography has not been utilised very successfully for the separation of lipids and so only impregnated papers were used in this work. Two types of commercially available adsorbent-impregnated papers were tried as well as one "reversed phase" system utilising paper impregnated with silicone fluid.

a) Silicic acid impregnated paper

The paper used was Whatman grade No. SG81 containing 22% of silica by weight. Various solvents were tried, all utilising ascending chromatography, to obtain separations of the pigments in the leaf extracts studied. All separations were carried out at room temperature in the dark, to avoid photochemical decomposition of the pigments.

The following solvents were tried:

Chloroform

Chloroform/methanol (50:10)

Chloroform/methanol (30:10)

Chloroform/methanol (20:10)

Hexane/acetone (90:10)

Hexane/acetone (80:20)

Hexane/acetone (60:40)

Hexane/benzene (50:50)

Hexane/ether (70:30)

Hexane/ether (30:70)

Hexane/n-propanol (94:6)

Of these only hexane/ether (30:70) and chloroform were of any use in separating the leaf pigments, but hexane/acetone (90:10) did give a fair separation of the quinones and the less polar pigments present.

b) Alumina impregnated paper

The paper used in this case was Whatman grade No. AH81 containing 7.5% of alumina by weight. Ascending chromatography, under similar conditions to those in a) above, was used in an attempt to separate the quinones present. The solvents tried were also as in a) above and a fair separation of the pigments was obtained with hexane/n-propanol (94:6). Benzene was also tried and a good separation of the quinones present was achieved with this solvent (cf. Lichtenthaler, 1964, using Schleicher and Schüll paper No. 288). The neotetrazolium chloride reagent described in 2)b)(v) above was used to detect the quinones present on such chromatograms.

c) Silicone fluid impregnated paper

Sheets of Whatman 3MM paper were immersed for about 30 seconds in a 5% (w/v) chloroform solution of Hopkins and Williams' silicone fluids MS-550 or MS-555 (manufactured by Midland Silicones Ltd.). The papers were dried at room temperature and then loaded with samples of leaf quinones eluted from TLC plates, together with reference quinones,

and placed in the chromatography tank containing the solvent to equilibrate for 15-20 minutes. The chromatograms were then developed, in the ascending direction for about 3¼ hours, in the solvent of n-propanol/water (4:1). The chromatograms were stained with the neotetrazolium chloride reagent by the procedure of Lester and Ramasarma (1959). The Rf values obtained could be used as a preliminary means of identification of the quinones present, since lists of Rf values of various naturally occurring quinones in this solvent system are given by Kegel, Henninger and Crane (1962) and Crane and Dilley (1963). These workers used a Dow Corning silicone fluid No. 550 but Midland Silicones Ltd. (1965) state that their MS-550 is a directly equivalent preparation.

EXPERIMENTAL (B)

5) DOCUMENTATION OF CHROMATOGRAMS

Documentation of thin layer chromatograms was achieved by the Neatan technique described earlier or, more commonly, by illuminating the plates from below and tracing the chromatograms; this latter method was also used for paper chromatograms. With the Azoflex tracing paper used, chromatograms on fluorescent layers could also be traced under incident short wave ultra violet (254nm), since this material was sufficiently transparent to light of this wavelength.

Wagener (1964) has described the photocopying of TLC plates using Agfa "Copyrapid" Negative paper and Zeitmen (1964) and Radin (1965) have recommended the use of blueprint papers for this purpose. In this work two types of copy papers were tried for photographic recording, "Diazochrome Blue" (Technifax) and Ilford "Azoflex". The Diazochrome material gave positive transparencies simply by developing with ammonia vapour (about 20 seconds) whilst the positive prints produced with the Azoflex paper required a short development in the special Azoflex developer. Since the Azoflex material required a shorter exposure time (about one third as long) this was used as standard. The photosensitive surface of the Azoflex paper was placed in contact with the adsorbent layer of the TLC plate illumination was provided through the plate by a 1000watt iodine

quartz lamp. At a distance of about 40cm exposures of 1½ to 1¾ minutes were required with 250μ silica gel plates. Under standard conditions the photocopies produced were suitable for densitometry.

Excellent resolution was obtained with this method and some compounds could be detected even when they were not easily visible on the plate. The sensitivity was greatest for yellow compounds since the Azoflex paper has a high sensitivity to blue light. Good results have been obtained with carotenoids, dinitrophenyl amino acids, amino acids (stained with ninhydrin), tocopherols (stained with phosphomolybdic acid or the diazo reagent), and general lipids stained with iodine vapour or sulphuric acid. With the last reagent the TLC plate was placed face-up instead of face down on the Azoflex paper and resolution was fractionally poorer with this method.

No attempts were made to record chromatograms under ultra violet light although Jones (1965) and Jackson (1967) describe methods for achieving this.

EXPERIMENTAL (C)

SPECTROPHOTOMETRY

Spectrophotometric measurements throughout this work were performed either on a Unicam SP500 spectrophotometer or a Unicam SP800 recording spectrophotometer. The directions for the use of these instruments are given in their respective instruction manuals and these directions were always followed exactly. Fused silica cells with a 1cm light path were used throughout most of this work.

1) ESTIMATION OF CHLOROPHYLLS

Chlorophylls were estimated spectrophotometrically by a modification of the method of Comar and Zscheile (1942).

Samples of leaf extract (dried over anhydrous sodium sulphate) equivalent to 0.5g of fresh leaf material, were taken to dryness under reduced pressure (only just, to avoid phaeophytin formation), dissolved in dry, peroxide-free ether and made up to 100ml. The optical density of this solution was measured at 660 and 642.5nm on the SP500 spectrophotometer.

The concentrations of chlorophyll a, chlorophyll b and total chlorophyll in the solution, in mg./litre, were calculated from the following equations (only applicable when, as in this case, the light path is equal to 1cm):

Chlorophyll a (mg./l) = 9.93 x Y - 0.77 x Z

Chlorophyll b (mg./l) = 17.6 x Z - 2.81 x Y

Total chlorophyll (mg./l) = 7.12 x Y + 16.8 x Z

Where Y = optical density of the solution at 660 nm

and Z = optical density of the solution at 642.5nm

Division of the results of each of these equations by five gave the chlorophyll (a, b or total) content in mg /g of leaf material. The chlorophyll contents in the Results Section are expressed as mg /100g of leaf, since this was found to be a more convenient reference value.

2) IDENTIFICATION AND ESTIMATION OF CAROTENOIDS

The bands of adsorbent containing carotenoids on the TLC plates were scraped off and eluted with ether as described in 2)a) of section B above. The extracts were taken to dryness under reduced pressure and the residues dissolved in hexane. The absorption spectra of these solutions were then determined. Absorption spectra of the carotenoids were also determined in ethanol solution

The carotenoids were identified by comparison of their absorption spectra with data in the literature on the basis of wavelengths of maximum and minimum absorption (e.g. Goodwin, 1955) and also by comparison of their relative log I/I_o values (optical density) at these wavelengths (Moster, Quackenbush and Porter, 1952; Bickoff, Livingston, Bailey and Thompson, 1954). Absorption spectra obtained, and their identification, are shown in the Results Section (figs 12-16).

The presence of epoxide groups was determined by recording the absorption spectra of the carotenoid, adding one drop of concentrated hydrochloric acid and recording

the absorption spectra. The wavelength of maximum absorption (in ethanol) is decreased by about 22nm for each epoxide group present (Goodwin, 1955).

For quantitative estimations of the carotenoids present, the bands of adsorbent from the TLC plate were eluted with ethanol, filtered and made up to a known volume with ethanol (20-50ml for each of the major carotenoids from about 4g of fresh leaf material). An exception was -carotene, which was eluted with ether, taken to dryness and then dissolved in a known volume (20-50ml as above) of hexane. The optical density of these solutions was then measured at their wavelengths of maximum absorption (determined as above). From these measurements and the dilution, the concentrations of various carotenoids in the leaf extracts could be calculated using the following specific extinction coefficient ($E^{1\%}_{1cm}$).

Neoxanthin (ethanol) : 2,270 (Strain, 1938a)

Violaxanthin (ethanol) : 2,550 (Karrer and Jucker, 1943)

Antheraxanthin (ethanol) : 2,420 (see below)

Lutein (ethanol) : 2,550 (Strain, 1938a)

β-carotene (hexane) : 2,650 (Zechmeister, 1944)

β-cryptoxanthin (hexane) : 2,460 (Goodwin, 1955)

α-cryptoxanthin (hexane) : 2,633 (Cholnoky, Szabolcs and Nagy, 1958)

Zeaxanthin (hexane) : 2,350 (Goodwin, 1955)

The specific extinction coefficient for antheraxanthin was calculated from the molar extinction value of 14.55×10^4

(calculated from data of Krinsky and Goldsmith, 1960).

The carotenoid values so obtained are tabulated in the Results Section both as mg/100g fresh weight of leaf material and as mg/100mg of chlorophyll; chlorophyll being measured on a replicate sample of the same leaf extract by the method in 1) above.

3) IDENTIFICATION AND ESTIMATION OF QUINONES

For the detection of quinones on the TLC plate a 2cm strip of adsorbent at the edge of the plate was sprayed with the neotetrazolium reagent (reagent (v) in B3) above), the rest of the adsorbent layer being covered with a clean glass plate. The position of the quinone-positive components on the plate was noted and then bands of unsprayed adsorbent corresponding to these positions were scraped from the plate with the aid of a spatula, and the quinones eluted with ether or ethanol. The ether/ethanol solutions were taken to dryness and dissolved in either hexane or ethanol for quantitative estimation. Quantitative estimation of phylloquinone, ubiquinone and the plastoquinones was performed by measuring the optical density of their solutions at the wavelength of maximum absorption. The concentration of these solutions could then be calculated from the following specific extinction coefficients:

ubiquinone-10 (hexane)	: 176)	(Isler, Rüegg, Langemann, Schudel, Ryser and Wursch, 1961)
phylloquinone (hexane)	: 422)	
plastoquinone A (hexane)	: 253)	

plastoquinone B (hexane) : 190 } (Calculated from above value for plastoquinone A, with a knowledge of the molecular weights.)

plastoquinones C and D (hexane) : 249 }

α-tocopherolquinone (ethanol) : 412 (Dilley and Crane, 1963b)

Reduction of ethanol solutions of the quinones was achieved by the addition of a few grains of potassium borohydride; the spectrum of the reduced quinone was then determined and the concentration was also calculated from the difference in optical density at the quinone's wavelength of maximum optical density. The following extinction coefficients were used ($E^{1\%}_{1cm}$, oxidised-reduced).

ubiquinone-10 : 142 (Lester, Hatefi, Widmer and Crane, 1959)

plastoquinone A : 198 (Morton, 1961)

plastoquinone B : 148 (Calculated from the above value

plastoquinones C and D : 194 (for plastoquinone A)

α-tocopherolquinone: 397 (Dilley and Crane, 1963b)

Unresolved mixtures of plastoquinone D and α-tocopherolquinone were assayed by the method of Bucke and Hallaway (1966). For this the change in absorption (on reduction) at 254nm and 262nm was measured in ethanol solution. The difference in these two values (change at 262nm minus change at 254nm) was then taken as 0.27 times the change at 262nm for α-tocopherolquinone. The concentration of α-tocopherol quinone was then calculated from the value of $E^{1\%}_{1cm}$ given above.

The concentration for plastoquinone D could similarly be calculated from the difference between the values of $E^{1\%}_{1cm}$ at 262nm for α-tocopherolquinone and the mixture.

EXPERIMENTAL (D)

ETIOLATION EXPERIMENTS

For the etiolation studies seeds of radish (Raphauus sativus var. Crimson giant) were planted in moist potting compost and grown in the dark at 20-25°C for five days. After this time the seedlings were about 6cm high with only their cotyledon leaves showing and they were then illuminated continuously at 18-20°C by means of a single 80 watt fluorescent tube ("daylight type") placed 130cm above them. 20.5g samples of the cotyledon leaves (with between 0 and 0.5cm of stem) were taken at intervals throughout the period of illumination and were extracted as described in Experimental Section A1)b). Each extract was water washed, made up to a known volume and a sample equivalent to 0.5g fresh weight was taken for chlorophyll determination. A volume equivalent to 5g fresh weight was taken and saponified (Experimental Section A 4)) and then subjected to TLC on silica gel, as described earlier, using hexane/acetone (60: 40) as the solvent in an unlined tank. The remaining leaf extract was washed with aqueous methanol and its quinone content was assayed by means of the column chromatographic method described in Experimental Section B 1).

The results of such etiolation experiments, with respect to the development of the leaf pigments and quinones with increasing time of illumination, are described in the Results Section. The concentrations of all the components are given as mg/100g fresh weight of leaf material.

EXPERIMENTAL (E)

ISOLATION OF PLASTOQUINONE-4

Plastoquinone-4 was required for chromatographic comparison with other quinones found in leaf extracts and was isolated from horse-chestnut leaves as follows. 1Kg of horse-chestnut leaves were added in 100g portions to 2.5 litres of ice-cold acetone in a large Waring blendor. After each addition the mixture was homogenised for one minute at low speed and then one minute at top speed. When all the leaf material had been thoroughly homogenised in the acetone, 800ml of cold n-hexane were added and the blendor was run at top speed for a further two minutes. The leaf homogenate was then filtered through Whatman No. 4 filter paper at reduced pressure and the residue was washed alternately with hexane and acetone until almost colourless.

The combined filtrate and washings were now extracted with 2.5 litres of water and 500ml each of 80% and 90% aqueous methanol. The green hexane extract remaining (approximately 2 litres) was then taken to dryness and the residue dissolved in 250ml 1,2-dichloroethane. This solution was then shaken with five 40g portions of Woelm Alumina (grade I) to remove chlorophyll and the polar lipids. Between each alumina treatment, the extract was filtered through a sintered glass filter and the alumina residue washed with 20ml fresh dichloroethane. The dichloroethane extract was now taken to dryness and the brownish oily

residue was dissolved in 200ml of warm absolute ethanol and allowed to stand at 0°C overnight.

The precipitate from the ethanol solution was filtered off and the solution was taken to dryness and dissolved in 100ml warm n-hexane. This solution was allowed to stand overnight at 0°C and then filtered and the orange-brown filtrate was taken to dryness and dissolved in 30ml warm acetone. The acetone solution too, was allowed to stand overnight at 0°C and then filtered and taken to dryness. The residue was then dissolved in 10ml warm n-hexane and loaded onto a 10 x 1cm column of Woelm alumina (Grade III). This column was eluted (under a pressure of about 25ml of solvent) with 25ml n-hexane, 50ml 10% ether/n-hexane and then 40ml 25% ether/n-hexane.

The 10% ether/n-hexane fraction was taken to dryness and strip loaded onto 30 20 x 20cm 500u Silica Gel H plates and these were developed in benzene in a lined tank. Detection with the neotetrazolium chloride reagent showed bands at Rf 0.5 (amongst others) and these were removed, eluted with ether, taken to dryness and dissolved in a little n-hexane. This solution was then rechromatographed on single 500u Silica Gel H plate in benzene and the fraction at Rf 0.5 eluted. This fraction was then chromatographed on a reversed-phase paraffin impregnated silica gel plate with 5% water/acetone in an S-chamber, to remove any plastoquinone B (Rf 0.25) and the band at Rf 0.8 was eluted as

plastoquinone-4. This plastoquinone-4 extract was finally purified by TLC on Silica Gel H in benzene to remove the liquid paraffin eluted from the reversed-phase plate.

EXPERIMENTAL (F)

PREPARATION OF TOCOPHEROLQUINONES

The method of Dilley and Crane (1963b), using gold chloride oxidation, was used to prepare samples of the tocopherolquinones for chromatographic comparison with compounds isolated from eleaf extracts. 0.1% ethanolic solutions of the authentic tocopherols (α, β, γ and δ) were prepared and to 1ml of each was added 0.2ml of a fresh aqueous solution of gold chloride containing 0.21g/ml. The solutions were shaken and allowed to stand in the dark for 30 minutes. After this time 20ml of n-hexane was added to each and the organic extracts were washed three times with 20ml portions of distilled water. The tocopherol-quinone solutions were then taken to dryness and the residues each dissolved in 1ml of hexane. TLC of these quinones with various solvents showed only a single quinone-positive spot for each and so no further purification was attempted.

EXPERIMENTAL (G)

PURIFICATION OF SOLVENTS

Solvents used for chromatography in this work were normally either Reagent grade or AnalaR grade, but for some purposes the solvents available required further purification before use. The main reason for the purification of such solvents was to obtain reliably standard results with respect to spectrophotometry and column chromatography of the quinones. Absolute ethanol gave satisfactory results for spectrophotometry but the M & B n-hexane used was not always so reliable (although better than any other tried). Since the majority of n-hexane grades available are contaminated with traces of aromatic compounds, with very strong short-wave ultra violet absorption, various attempts were made to remove such compounds.

The recommended techniques for the removal of aromatic hydrocarbons are shaking with either aqueous potassium permanganate or concentrated sulphuric acid, but neither of these procedures removed the traces of aromatics shown by the ultra violet spectrum. Chlorosulphonic acid was also tried, but since a rather violent reaction ensued, this was discontinued. Passage of solvents through activated alumina can often give at least partial purification and a 12 x 12cm column of Woelm Alumina (grade I) was found to decrease the ultra violet absorption of n-hexane slightly. In view of this, such a column was used routinely to purify

all n-hexane used in this work and a single column was normally sufficient for about 15 litres (the first 50ml being discarded). The major compound removed by the alumina was orange-yellow with a spectrum showing little specific aromatic absorption but a strong broad peak with a maximum around 322nm. On standing on the alumina column with exposure to light, this orange-yellow compound turned purple. The absorption spectrum of the column effluent however, appeared to have lost a fraction with an absorption maximum at about 267nm and a shoulder at 275nm.

Redistillation of the column effluent either fractionally or from concentrated sulphuric acid, did not lead to any further purification of the n-hexane as shown by its ultra violet absorption spectrum and so in further work the n-hexane was used direct from the column.

The other solvent used in this work was 1,2-dichloroethane and the M & B product was again preferred, although BDH material showed very similar properties. To purify the dichloroethane, 3 litres were refluxed for 30 minutes with 200ml concentrated sulphuric acid and the solvent was then distilled off, the first 100ml being discarded. The distillate was fairly acid due to the presence of hydrogen chloride together probably with some sulphur dioxide. Various bases and indicators were tried in order to achieve a method of measuring this acidity and a titration method was devised which gave reproducible results. Thymol blue was used as

the indicator and two drops of a 0.05% solution in absolute ethanol were added to 5ml of the dichloroethane in a small dry flask. 0.001 N potassium hydroxide in absolute ethanol was used to titrate this solution, the indicator colour change being from red to yellow under these conditions.

The titre obtained with the distilled dichloroethane above was usually about 30ml .001 N alkali for a 5ml sample; this is equivalent to an acid content of about 2ppm by weight. By bubbling pure dry nitrogen through this solvent for 5-10 minutes the acid content could be reduced to less than 0.1 ppm (a titre of 1.5ml per 5ml sample). Consequently, nitrogen was bubbled through all dichloroethane used in this work (after distillation from sulphuric acid) until its content was less than 0.1 ppm.

RESULTS SECTION

RESULTS (A)

PREPARATION AND INITIAL ANALYSIS OF LEAF EXTRACTS

All the methods used for extraction of the leaf material gave similar results as regards chlorophyll and carotenoid content (see Section C), although as regards total lipid, acetone-insoluble lipid and saponifiable and unsaponifiable fractions, the results obtained from different leaves gave greatly differing values, independent of the method of extraction used; this variation is, to some extent, expected in plant materials and is probably due to differences in age, season or species. These values are shown in table 1 and are expressed as g/100g of fresh leaf material.

As stated in the Experimental Section, method b) was found to be the most convenient method of extraction, and it was probably the most efficient. Method c), utilising extraction with $^{N}/_{10}$ sodium hydroxide, gave similar results to the other two methods used and some results for the fibre and protein content of the leaf material used were also obtained with this method of extraction. These latter results, together with the total lipid content of the leaf material, are recorded in table 2.

TABLE 1 Analysis of leaf material for various lipid fractions.

Leaf material*		Total lipid	Acetone-insoluble fraction	Acetone-soluble fraction
Broccoli	(b)	3.25	1.16	2.09
Broccoli	(b)	2.35	0.12	2.23
Broccoli	(c)	1.53	0.87	0.66
Broccoli	(c)	1.11	0.23	0.88
Brussels sprout	(b)	1.39	0.40	0.99
Cabbage (young)	(b)	0.75	0.14	0.61
Cabbage	(c)	1.50	0.36	1.14
Cabbage	(c)	1.46	0.78	0.90
Cabbage	(b)	1.37	0.54	0.83
Kale	(c)	1.09	0.15	0.94
Spinach beet (young)	(b)	0.28	0.05	0.23
Spinach beet	(b)	0.97	0.24	0.73
Sugar beet	(c)	1.38	0.15	1.23
Sugar beet	(a)	1.02	0.19	0.83
Sugar beet	(a)	0.98	0.14	0.84

* (a), (b) or (c) refer to the method of extraction.

All values as g/100g fresh leaf material.

TABLE 2 Total Lipid, protein and fibre content of leaf material.

Leaf material	Total Lipid	Crude Protein	Fibre
Broccoli	1.53	5.30	8.10
Broccoli	1.11	3.86	6.52
Cabbage	1.50	4.25	7.25
Cabbage	1.46	5.80	10.60
Sugar beet	1.46	4.00	3.03
Sugar beet	1.38	5.01	3.45

All values as g/100g of fresh leaf material.

Conditions for the saponification of the chlorophyll in leaf extracts.

The minimum time for saponification of the chlorophylls present in leaf extracts, under the conditions described in the Experimental Section, was found to be in the region of 2-5 minutes with adequate shaking. For routine experiments the saponification was allowed to continue for 10-15 minutes, as this time ensured that all the chlorophylls present were converted into water soluble derivatives. In this short time, at room temperature, very little saponification of any of the neutral lipids of the leaf would be likely to occur.

Recovery of saponified chlorophyll derivatives from their aqueous solutions.

As described in the Experimental Section, three different adsorbents were tried for the removal of saponified chlorophylls from their neutral aqueous solution (pH6).

The results of these experiments were as follows:

Silicic acid: this adsorbent removed the chlorophyll derivatives from solution fairly efficiently and these could be eluted from the silicic acid with acetone, or, more rapidly, with methanol.

"Low temperature burnt magnesia": this adsorbent removed the chlorophyll derivatives from solution very efficiently, but the adsorbed material could not easily be eluted, even with methanol or chloroform/methanol (1:1).

Calcium phosphate: this was not a very efficient adsorbent for the saponified chlorophylls and the little of the material that was adsorbed could not be eluted with acetone and was only very slowly eluted with methanol (i.e. large volumes of methanol required).

RESULTS (B)

SEPARATION OF THE LEAF LIPID COMPONENTS

1) COLUMN CHROMATOGRAPHY

As stated in the Experimental Section, the pigment separations obtained by column chromatography were not very satisfactory, although the separation of a leaf extract on a silicic acid column, using increasing concentrations of acetone in n-hexane, did show the presence of an orange carotene fraction together with three major and two minor yellow xanthophyll components. Better separations of the quinones were obtained however, and the procedure described in the Experimental Section was developed as a partially automated system for the routine separation and estimation of the leaf quinones.

Fig. 31 shows the Uvicord recorder trace of a typical leaf quinone separation as a function of transmission of the column effluent against time, and table 3 shows the identification of the peaks appearing on this trace. These compounds were identified as described in later sections by means of their chromatographic behaviour in various systems and by comparison with the literature and with authentic compounds where these were available. Also shown in fig. 31 is the gradient (of dichloroethane in n-hexane) used to give the elution pattern shown. It should be noted that this is the gradient applied to the top of the column and factors such as the delay due to the solvent

Column separation of leaf quinones

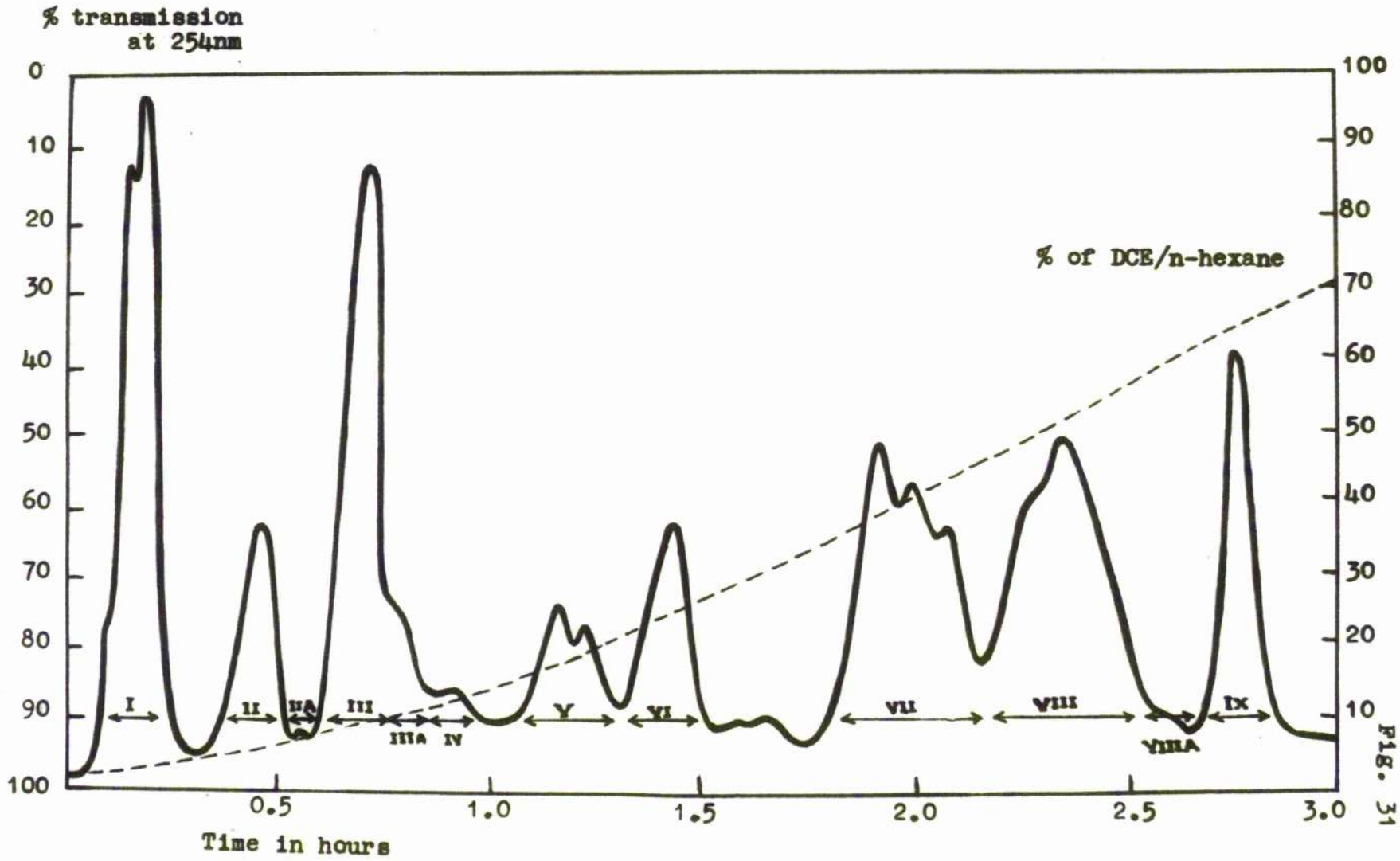


Fig. 31

volume of the column (about 18ml) and the effects of the adsorbent on the concentration gradient are not taken into account.

TABLE 3 Identification of fractions in fig. 31

Peak No.	Identification	Comments
I	Waxes and terpenoid hydrocarbons such as squalene; β-carotene	Orange
II	Phylloquinone	-
IIA	Demethylated naphthoquinone	Only occasionally present
III	Plastoquinone A	Large peak
IIIA	Lower isoprenologue of plastoquinone A	Usually tail on peak III
IV	Plastoquinone B-type compound	Not always resolved
V	Plastoquinones B	Usually partially resolved into two peaks
VI	Ubiquinone-10	-
VII	Plastoquinones C	Resolved into three peaks
VIII	Plastoquinones D plus α-tocopherolquinone	Occasionally resolved into more than one peak
VIIIA	β+γ-tocopherolquinones	Slight peak usually seen
IX	Phaeophytin	-

Various problems were encountered during the development of this routine column chromatographic method, the first, obviously concerning the choice of adsorbent and solvents. As stated in the Experimental Section, promising

separations of the quinones could be achieved on alumina and reasonable flow rates were also obtainable using this adsorbent. Links (1960) has reported the conversion of ubiquinone into its cyclic isomer ubichromenol on columns of activated alumina, and Bats, Gervais and Cofc (1962) have also reported the conversion of various quinones to their chromenols on a number of different adsorbents including alumina. Hemming, Morton and Pennock (1961) however, concluded that little alteration occurred during short periods of time on partially deactivated alumina and, as a consequence, alumina used in this work was deactivated to Brockmann Grade III by the addition of 6ml water to each 100g.

Various non-polar solvents were available and n-heptane and cyclohexane were tried in addition to n-hexane. Both these solvents gave lower flow-rates than n-hexane under the conditions used and this effect is probably due to the higher viscosity of these other hydrocarbons. As stated in the Experimental Section, the n-hexane used was checked to ensure that it was reasonably free from ultra-violet absorbing impurities. The polar solvent chosen for the concentration gradient was 1,2-dichloroethane, the reason being that this solvent was obtainable in a fairly pure state and free of aromatic impurities. Solvents such as ethyl acetate, chloroform and acetone were unsatis-factory since their optical density at 254nm (the Uvicord

monitoring wavelength) was too high and the highly polar alcohols exhibited discontinuous adsorption behaviour with alumina. Ether was not used as the solvent in this work, since its low boiling point would probably have led to disturbance of the column packing; for the same reason pentane was unsuitable as the non-polar solvent. Where necessary the optical density of the dichloroethane at 254nm was made equal to that of the n-hexane by the addition of a minute trace of AnalaR benzene.

The gradient elution system developed is described in the appropriate section and as reported in the Experimental Section, Durrum "positive displacement tubing pump" was used to produce the gradients. The gradient shown in fig. 31 appeared to give similar separations independently of the mass of leaf extract used. Shallower gradients of the same general shape gave satisfactory results for the extracts from larger weights of leaf, although they were unsuitable for smaller amounts. This was probably due to a "displacement analysis" effect with the larger amounts. The pumps originally tried in this work were DCL Micro pumps of the piston-type but these behaved erratically with the particular organic solvents used. Although these pumps normally operated satisfactorily with n-hexane, even low concentrations of dichloroethane caused the pumps to slip and to deliver decreased and irregular volumes. This did not appear to be due to particulate matter as the solvents

used were checked beforehand. Also the addition of even 2% of methanol corrected this behaviour. A Milton Roy pump of similar design exhibited the same behaviour as the DCL models and it seems possible that this may have been due to adsorption of the dichloroethane onto the stainless steel balls and seatings of the pump valves. For these reasons the piston pumps were replaced by the Durrum tubing pump as described.

The Durrum pump had one disadvantage in this work and this was its alteration of flow rate with the increasing back pressure during the development of the concentration gradient. This was caused by the increasing viscosity of the eluting solvent due to dichloroethane having a higher viscosity than n-hexane. This was counteracted to some extent by making use of the volume measurement provided by the event marker, rather than a time measurement (or distance on the recorder chart) in quantitative work. The method could be further improved by either altering the recorder chart speed with the changing flow rate (if this were possible), or else by finding a piston-type constant speed pump which would function properly with dichloroethane; the Milton Roy pump fitted with sapphire valves may be suitable for this.

As can be seen in fig. 31, the column technique described gives a satisfactory separation of the majority of the plant quinones. Better resolution could not be

obtained with either a shallower gradient or with lower flow rates although the pulsatile action of the pump may be partly responsible for this. Preliminary experiments suggest that better resolution may be obtainable using larger columns but this could not be investigated further in this work as the Durrum pump did not give high enough flow rates with the back-pressure developed with longer columns. The back pressure on the 100cm columns used appeared to be around 15lb/sq inch, but according to Vestergaard and Sayegh (1966), piston-type mini pumps can overcome a back pressure of up to 900lb/sq inch in such systems. Thus, if a suitable pump of the piston-type can be found it should be possible to achieve improved resolution by the use of longer columns.

The poor separation of the plastoquinone D group and α-tocopherolquinone shown in fig. 31 is not affected by the length of the column and it appears to be caused by the presence of steroid in the leaf extracts examined. Various procedures were tried to remove this steroid including a number of methods of digitonin precipitation and modifications to the column adsorbent. None of these were successful so the poor separation was accepted and the bulk fraction collected and assayed separately by the method of Bucke and Hallaway (1966) as described in the Experimental Section C 3). By comparison with TLC, somewhat anomolous results were observed in the behaviour of this steroid and α-tocopherolquinone.

On TLC with Alumina G both the steroid and α-tocopherol-quinone ran behind phaeophytin, whilst on the column, as can be seen, these compounds ran ahead of phaeophytin together with plastoquinone D. Incorporation of 10% gypsum into the column adsorbent did not alter the separation appreciably, and so the difference does not seem to be due to the binder present in the TLC material.

During the attempts to remove the steroid from leaf extracts a crude chloroplast suspension was prepared to see if this contained less steroid than the whole extract. Differential centrifugation of a "tris-sodium chloride" buffer extract was used and the "1000g" precipitate was collected. Column chromatographic analysis of the lipid extract of this fraction did not show any evidence of a decreased steroid level but the ubiquinone peak was present at approximately its normal concentration, thus showing that the 1000g chloroplast fraction was heavily contaminated with mitochondria.

Peak IIA shown in fig. 31 was observed in about 15% of the chromatograms run and from the few spectral results obtained it appears to be the naphthoquinone reported by McKenna, Henninger and Crane (1964) which they suggest may be a "dimethylated form of vitamin K,". No correlation was found between the occurrence of this compound and any factor such as light intensity, season or age of leaf. Peaks IIIA and IV in fig. 31 appear to be a mixture of at

least one plastoquinone B-type compound and another quinone which behaved, on reversed phase chromatography, as the lower isoprenologue of plastoquinone A, plastoquinone-8. Whether peak IIIA is due mainly to this compound or mainly to a B-type of quinone is uncertain. Some evidence was also seen for traces of a quinone with the expected properties of plastoquinone-7, but this could not be confirmed. Similarly, during the development of the correct gradient some irregularities were observed in the first part of the plastoquinone A peak which could have been due to the presence of small amounts of a higher isoprenologue such as plastoquinone-10.

RESULTS (B)

2) THIN LAYER CHROMATOGRAPHY

Good separations of the lipid extracts were obtained by the use of TLC and this technique was used for both whole leaf lipid extracts and for the further purification and identification of the column fractions reported in section A. Satisfactory separations of both pigments and quinones were achieved and various different adsorbents were tried, as described in the Experimental Section in an attempt to produce the best possible resolution in these separations.

Binder free silica gel (mainly Silica Gel H) and Silica Gel G were the adsorbents most widely used in this work and, as far as can be seen, they appear to give the best resolution of both pigments and quinones. Alumina G and alusil layers gave superior separations in some specific cases, notably in the separation of plastoquinone A and the compound presumed to be plastoquinone-8. Phylloquinone and plastoquinone A could also be separated on alumina. As a rule the adsorbents gave thin films of fairly reproducible properties when these were prepared by the procedure described in the Experimental Section. A notable exception was one batch of plates prepared from Whatman SG41 (binder free silica gel). Although the same procedure was followed as for the preparation of other plates from SG41, this particular preparation produced adsorbent layers of rather

unusual properties. The film of adsorbent on these plates appeared to have exceptional cohesive properties, but the rate of solvent travel was much slower than usual (e.g. a distance of 12cm, normally requiring 30-40 minutes, required over two hours) although similar Rf values were obtained. These effects may have been caused by some unrecognised abnormality in the drying procedure which caused the adsorbent particles to compact together, although no reference to any such effect has been found in the literature.

The degree of activation of the silica gel layer was found to have a great effect on the Rf values obtained, but not on the rate of solvent travel (contrast the effect found with SG41 above). Best separations were found to be obtainable on highly active plates and these were prepared by allowing minimal contact with the atmosphere after drying at 110°C. The plates had to be allowed to cool to at least 40-45°C (to prevent destruction of pigments and quinones) and this was preformed in a dessicator when possible. Plates were stored in a dessicator over blue silica gel until required, often for long periods, and this did not appear to have any effect on their subsequent properties.

Silica Gel G/UV-254 was not very successful for the purpose for which it had been tried, namely for the detection of quinones. Under ultra-violet light, compounds absorbing such wavelengths appear as dark spots on a fluorescent ground with this adsorbent. The sensitivity of this

method of detection appears to be very low however, and only one or two adsorbent bands were visible under ultra violet light, in a region of the chromatogram in which the neotetrazolium reagent (v) revealed the presence of seven or eight quinonoid compounds.

Aluminium Oxide G was used for a number of separations, mainly of the quinones, and the results obtained suggest that although silica gel is slightly superior for general work, some specific separations of quinones can more easily be obtained on alumina. Davidêk and Blattnâ (1962) have reported the separation of the fat soluble vitamins A, B_2, E, K, K_2 and K_3 and α- and β-carotene on loose layers of alumina (Brockman Activity III-IV), using solvents of "petrol", petroleum ether and carbon tetrachloride, although according to Links (1960) activated alumina may cause the alteration of some of the quinones present.

It was observed in the initial pigment separations, that some samples of leaf extract gave very poor separations, with irregular bands of pigment on the TLC plate; these were somewhat similar to some of the separations obtained by Grob, Eichenberger and Pflugshaupt (1961). It was found that removal of the waxes and phospholipids from the extract appeared to prevent this unevenness and enabled highly reproducible separations to be obtained. Readdition of these acetone-insoluble lipids to the partially purified extracts was shown to produce poor separations thus

establishing that the unevenness and poor reproducibility of the chromatograms was due to this acetone-insoluble fraction. Thus, extraction of the lipid residue with acetone was introduced as a routine purification prior to TLC of the leaf extracts, the acetone solution being free of these "interfering lipids".

The weight of leaf extract strip loaded onto 250μ layers on 20 x 20cm plates was about 20mg, equivalent to 30-40mg of total leaf extract or 3-4g of fresh leaf. On 500μ layers of binder free silica gel, about 50mg of partially purified extract could be satisfactorily separated.

a) Separation of the pigments in leaf extracts

As stated in the Experimental Section, the best solvent for the separation of the leaf pigments on silica gel was found to be hexane/acetone (60:40) although hexane/acetone (50:50) gave good resolution of the xanthophylls. The acetone used was normally dry AnalaR acetone; the hexane was also dry and of fairly high purity. As expected, best results were obtained by using dry solvents on activated plates.

Sample separations of the pigments in normal and saponified leaf extracts (in hexane/acetone (60:40) on Silica Gel G) are shown in fig. 32. Fig. 33 shows a similar TLC (but spot loaded, not strip loaded) after spraying with a saturated solution of antimony trichloride in chloroform (see the "Carr-Price" test in 2 d) ii) of the Experimental Section).

Fig. 32

TLC OF LEAF EXTRACT

Silica Gel G

Solvent: n-hexane/acetone (60:40) (unlined tank)

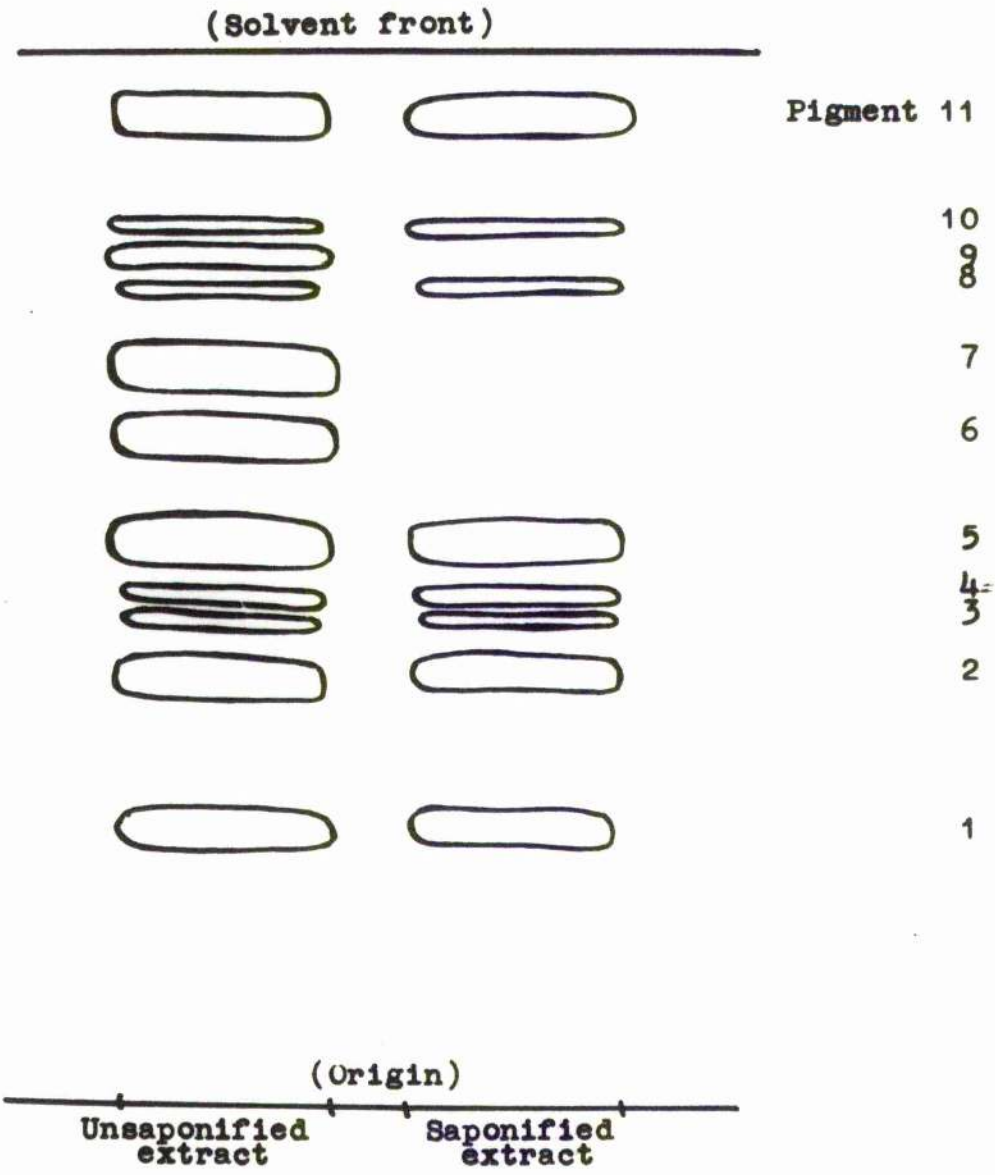
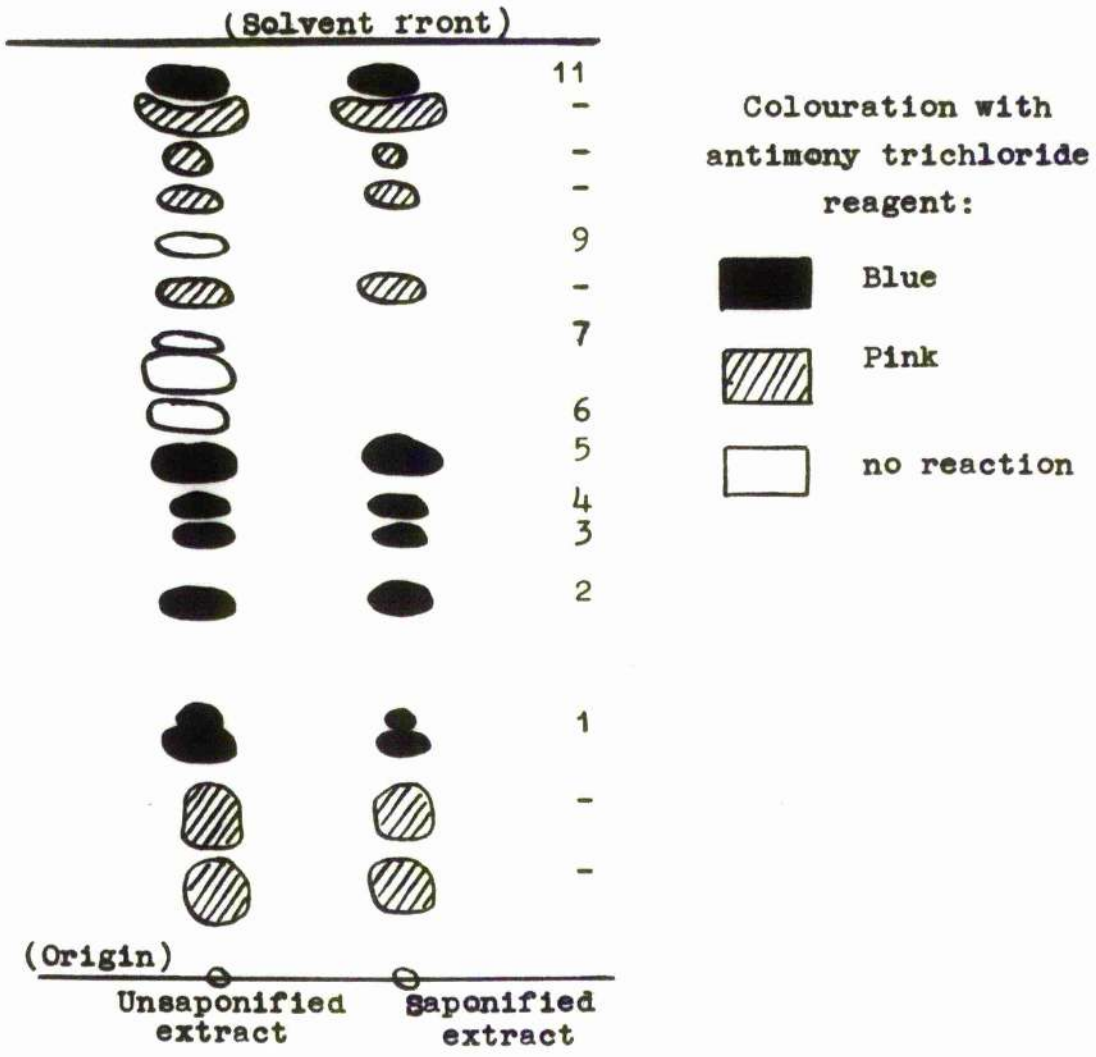


Fig. 33

TLC OF LEAF EXTRACT

Silica Gel G

Solvent: n-hexane/acetone (60:40)(unlined tank)



The pigment fractions were allotted numbers from 1 to 11 but it should be noticed in fig. 33 that fraction 1 may contain a trace of another pigment. The results of these separations as regards colour, occurrence, approximate amount and reaction with antimony trichloride are recorded in table 4 following.

TABLE 4 Analysis of figures 32 and 33

Pigment number	Colour	Relative amount	Occurrence	Reaction with antimony trichloride	Initial identification
11	Orange	Much	Always	Blue	Carotene
-	-	?	Always	Pink (several spots)	Steroid esters and quinones
10	Pale Orange	Little	Always	?	Xanthophyll
9	Grey	Moderate	Usually	-	Phaeophytin
-	-	?	Always	Pink	Steroid
8	Yellow-Orange	Trace	Usually?	?	Xanthophyll
7	Dark-green	Much	Always	-	Chlorophyll a
6	Yellow-green	Much	Always	-	Chlorophyll b
5	Orange-yellow	Much	Always	Blue	Xanthophyll
4	Yellow	Little	One always, the other occasionally	Blue	Xanthophyll
3	Yellow	Little		Blue	Xanthophyll
2	Yellow	Moderate	Always	Blue	Xanthophyll
1A	Pale Yellow	Trace	Occasionally	Blue (slight)	Xanthophyll
1	Yellow	Moderate	Always	Purple-blue	Xanthophyll
-	-	?	Always	Pink (2 spots)	Glycolipids

As can be seen there are a number of colourless fractions which gave the pink colour typical of steroids with antimony trichloride/chloroform. Later work however, shows that several of these compounds were quinones which may also give a pink or brownish colouration on heating with this reagent. On the chromatogram in fig. 33 the two yellow-orange bands running on either side of phaeophytin did not appear to give a blue colouration with the Carr-Price reagent. Later evidence showed that pigment 10 at least did give a blue colouration, but it may not have been in high enough concentration to show on the chromatogram in fig. 33. Spraying with Rhodanine (spray reagent (iii) in the Experimental Section) gave no red colourations, thus showing the absence of any carotenoid aldehydes.

In fig. 33 an additional green spot running ahead of chlorophyll a can be seen; this was observed on a number of chromatograms and probably corresponds to the allomerisation product of chlorophyll a, chlorophyll a' found by Strain and Manning (1942). In a small number of separations an apparently similar effect was observed in which chlorophyll a and b each separated into two or three spots which sometimes showed a tendency to "tail" and partially obscure some of the xanthophylls. The cause of this effect is uncertain, although it may represent a conversion of the chlorophyll into the types of pigment reported by Michel-Wolwertz and Sironval (1965) as naturally occurring forms of chlorophyll

in Chlorella vulgaris. Bacon (1966) concludes that such pigments may be artefacts formed by oxidation of the native chlorophylls, either during the preparation of leaf extracts or during chromatography. In agreement with this it should be noted that this multiple zoning of the chlorophylls was not observed when 0.1% of the antioxidant BHT (4-methyl-2,6-ditertbutyl-phenol) was incorporated in the developing solvent, as suggested by Wren and Szczepanowska (1964). It is also of interest that no change in the carotenoid separations was observed in the presence of this antioxidant and so the pigments found do not appear to be oxidation artefacts formed during chromatography.

The carotenoid fractions 3 and 4 were only observed in some of the leaf material examined and other extracts, particularly those of leaves gathered during the winter, were only found to contain one pigment running in this position on the TLC plate; this appears to correspond to pigment 4 rather than pigment 3. Other minor yellow or orange bands were sometimes observed running between pigments 10 and 11 but these were variable in both number and occurrence.

Several of the xanthophylls (pigments 1-4) were found to exhibit an unusual reaction when left in contact with the adsorbent after separation. The yellow xanthophyll was converted to a blue compound which, on reduction with potassium borohydride, was changed back to yellow, thus suggesting that the initial reaction was one of oxidation, probably

similar to the reaction of some carotenoids with concentrated sulphuric and hydrochloric acids (see Goodwin, 1955).

b) Separation of the quinones in leaf extracts by TLC

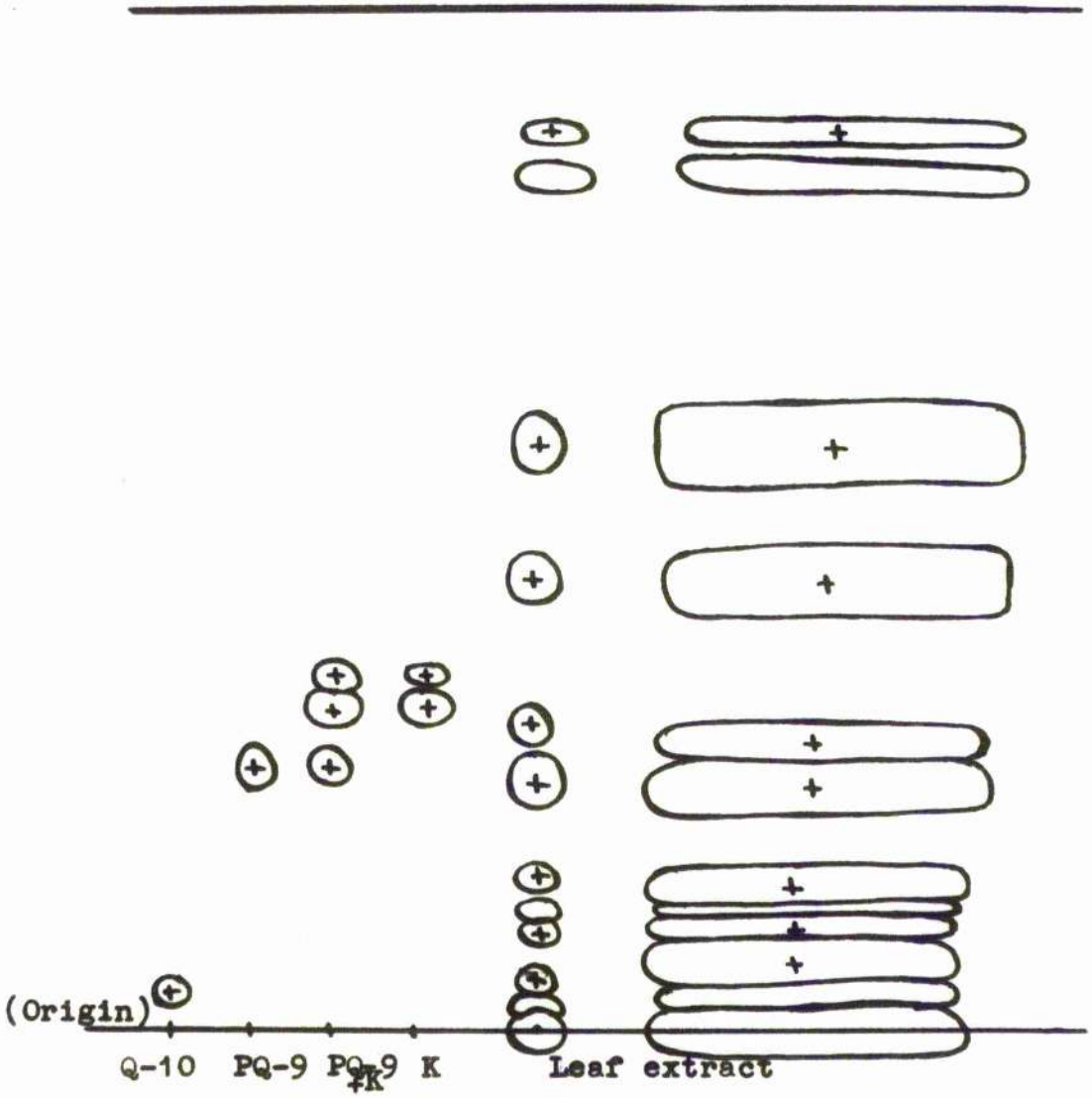
Separation of the quinones was achieved on Silica Gel H and Silica Gel G plates using solvents of fairly low polarity such as benzene and hexane/benzene mixtures. For the polar quinones such as the plastoquinones C and D and the tocopherolquinones, more polar solvents such as chloroform and dichloroethane were used. Figs. 34, 35 and 36 show representative separations of leaf extract on Silica Gel H plates, using solvents of hexane/benzene (50:50), benzene and dichloroethane respectively. All these separations were performed in an "S-chamber" in the dark; the effect of the S-chamber as compared with a normal tank, was to give slightly better resolution, lower Rf values and more even separations, even although the band width was increased slightly. The plates were sprayed with the neotetrazolium chloride as described in the Experimental Section (B 2)d)(v)) and the compounds observed, together with the intensity of their reaction with this reagent, are summarised in table 5 following. Although Rf values are given in this table, they can only be taken in the context of "order of running" on the chromatogram, not as an indication of actual reproducible position as in paper chromatography, because in TLC Rf values are subject to much more variation.

TLC OF LEAF EXTRACT

Silica Gel H

Solvent: hexane/benzene (50:50)(S-chamber)

+ : positive colouration with the neotetrazolium chloride reagent



TLC OF LEAF EXTRACT

Silica Gel H

Solvent: benzene (S-chamber)

+: positive colouration with the neotetrazolium chloride reagent

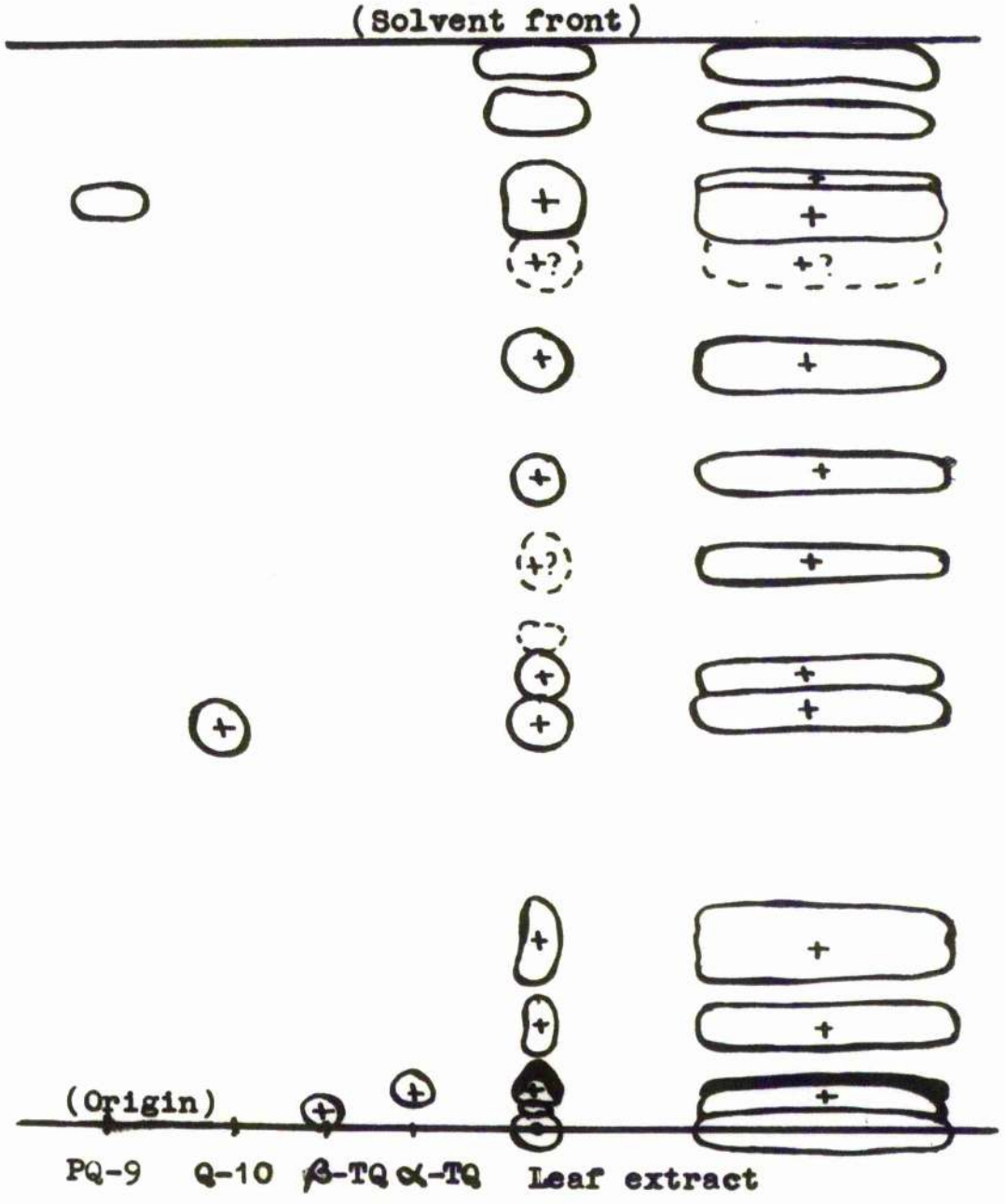


Fig. 36

TLC OF LEAF EXTRACT

Silica Gel H

Solvent: dichloroethane (S-chamber)

+: positive colouration with the neotetrazolium chloride reagent

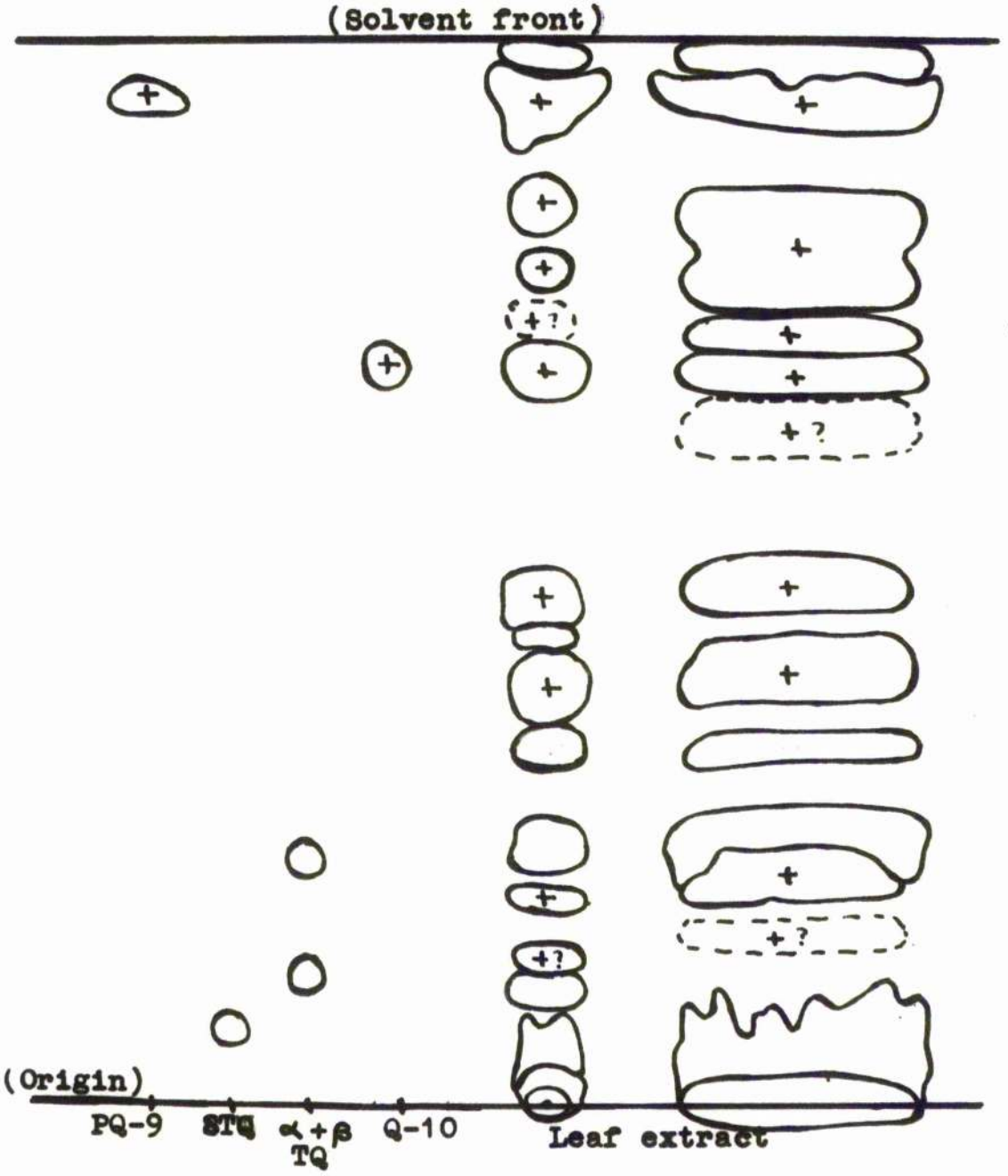


TABLE 5 Analysis of the leaf lipid separations shown in figures 34, 35 and 36.

"Rf value" fig.34	"Rf value" fig.35	"Rf value" fig.36	Strength of quinone reaction	Identification
0.89	-	-	++	Unknown
0.85	0.98	0.99	-	Carotene
0.58	0.92	-	+	Wax-like fractions
0.45	0.92	-	+	
0.29	0.86	0.95	++	Phylloquinone
0.25	0.86	0.95	+++	Plastoquinone A
0.16	0.72	0.92	++	Plastoquinone B-type compounds
	0.61	0.85	++	
	0.53	0.79	+	
-	0.42	0.73	+?	α-tocopherol or plastoquinol A?
0.10	0.38	0.69	++	Ubiquinone-10
Origin	-	0.63	?	Unknown, possibly β or γ-tocopherol
Origin	0.18	0.48	+++	Plastoquinone C-type compounds
Origin	-	0.44	-	Carotenoid
Origin	0.11	0.40	++	Plastoquinone D-type compounds
Origin	0.06	0.34	-	Carotenoid
Origin	0.04	0.24	-	Phaeophytin
Origin	Origin	0.20	++	α-tocopherolquinone
Origin	Origin	0.14	+	$\beta+\gamma$-tocopherolquinones
Origin	Origin	Origin	-	Chlorophylls + xanthophylls

The identifications in table 5 are based on cochromatography with authentic compounds (where these were available) and also on comparisons with the literature and on spectrophotometry (see Results Section C). Two wax-like fractions which gave a positive reaction with the neotetrazolium chloride reagent are shown in fig. 34 and on attempted purification of these they were found to be somewhat unstable (these are further discussed in part c) following).

The separation of authentic phylloquinone into two spots shown in fig. 34 was only observed on binder-free silica gels and the best resolution was actually achieved on Whatman SG41 with a solvent of hexane/benzene (50:50) in an unlined tank. The observed separation is not due to "double zoning" as the separate spots could be eluted and ran with their original Rf values on rechromatography. Mayer, Gloor, Isler, Rüegg and Wiss (1964) reported that authentic phylloquinone contained both 3'-cis and 3'-trans isomers which were just separated by TLC on Silica Gel G with hexane/n-butyl ether (92:8) as the solvent and a similar separation has obviously been achieved in this case. Mayer et al report that the 3'-cis phylloquinone runs ahead of the 3'-trans isomer and, as expected, it can be seen that the natural phylloquinone runs with the same Rf as the synthetic trans form. This was found to apply both to whole leaf extracts and to fraction II isolated by the gradient elution chromatographic method described. When fraction IIA was

found, it ran with the same Rf as phylloquinone.

Only a slight separation between plastoquinone A and phylloquinone can be seen in fig. 34 and no other solvent system tried on silica gel could improve on this separation (most did not give any separation at all) although TLC on alumina using 5% dichloroethane/cyclohexane gave Rf values (on the same plate) of 0.66 for phylloquinone and 0.54 for plastoquinone A.

The plastoquinone A isolated from leaf extracts cochromatographed exactly with synthetic plastoquinone-9 (obtained as a gift from Hoffman-La Roche of Busle) both on normal TLC and in reversed phase techniques (see Results Section A 3)a) and a faintly quinone-positive fraction was sometimes observed in leaf extracts which ran just below the major plastoquinone-9 fraction in normal (adsorption) TLC. This fraction appears to correspond to a mixture of fractions IIIA and IV from the gradient elution separations. This minor fraction was better separated from plastoquinone-9 by TLC on alumina layers using hexane/ benzene (80:20) as the solvent; the Rf values in this system were 0.34 and 0.42 respectively.

The three plastoquinone B components shown in figs. 35 and 36 were not always all visible in TLC separations of whole leaf extracts, but this was probably a concentration effect since they were found to correspond to fraction V in the column separations and fraction V was always observed.

Although the Rf values of the three plastoquinone B components varied, their Rfs with respect to a standard such as -tocopherol were relatively constant using benzene as a solvent on silica gel in an S-chamber. The R_{α} values (Rf of the compound divided by the Rf of α-tocopherol) for the three plastoquinone B components were 1.65-1.70, 1.45 and 1.25 in the above system and the values for plastoquinoneA and its slower running contaminant were 2.00-2.10 and 1.90-1.95 respectively. The first peak in the column fraction V corresponds to the faster running B-component whilst the second peak is composed of the two slower ones.

The faint quinone-positive fraction running ahead of ubiquinone-10 in leaf extracts probably corresponds to α-tocopherol or, in some cases plastoquinol A since this compound (prepared by the reduction of authentic plastoquinone A) ran in this position. α-tocopherol also ran with the same Rf and was found to give a slight reaction with the neotetrazolium chloride reagent under some conditions. Plastoquinol A was only rarely observed in leaf extracts, probably because the extraction methods used would reoxidise any quinols present to their quinone forms. The ubiquinone fraction was present in leaf extracts and cochromatographed with ubiquinone-10. This quinone was normally the only one present in fraction VI in the column chromatographic separations although traces of one of the plastoquinone B components (R_{α} = 1.25) were sometimes present.

Only two quinone-positive spots or bands were normally observed running between ubiquinone and phaeophytin in leaf extracts, although these spots (or bands) were often somewhat elongated. The R_{α} values of these quinones were approximately 0.41 and 0.28 (on silica gel with benzene as the solvent) and they appear to correspond to plastoquinones C and D respectively. TLC of fraction VII from the column separations did reveal the presence of three components but these were usually fairly diffuse with most systems tried. TLC on alusil layers, with dichloroethane as the solvent, however, did give fairly sharp separations and under such conditions the three plastoquinone C components ran between the γ- and δ-tocopherols used as markers. TLC of the column fraction VIII under the same conditions normally showed the presence of five quinone-positive components, two of which cochromatographed with α- and β/γ-tocopherolquinones. The other three components ran with higher Rf than α-tocopherolquinone, but below plastoquinone C and appear to correspond to the three components of plastoquinone D. Fig. 37 shows a separation of these compounds on alusil with dichloroethane as the solvent. Quinones with similar Rf values to the plastoquinone C and D components were sometimes observed during rechromatography of the plastoquinone B group and this may have been due to the removal of the esterifying fatty acids by hydrolysis.

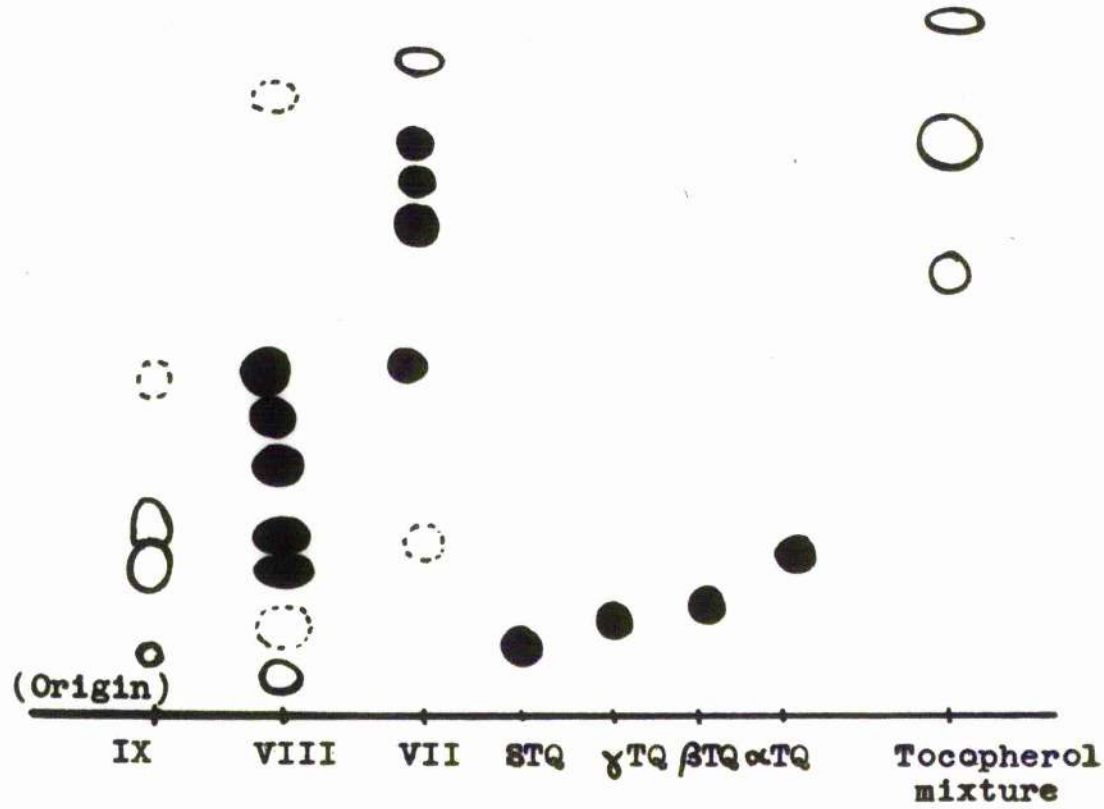
TLC separations of the six plastoquinones C and D components in whole leaf extracts could sometimes be achieved

TLC OF COLUMN FRACTIONS

Alusil (50:50)

Solvent: dichloroethane (unlined tank)

positive reaction with the neotetrazolium chloride reagent



on alumina layers with hexane/dichloroethane mixtures as solvents. The presence of -tocopherolquinone was also demonstrated in such chromatograms as well as on silica gel, together with a smaller quinone-positive fraction which appeared to be either - or -tocopherolquinone. -tocopherolquinone was never observed either in whole leaf extracts or during TLC of column fractions.

c) TLC of other compounds observed in leaf extracts

A large number of other components apart from quinones and pigments were observed in leaf extracts by TLCof both whole leaf extracts and of column fractions. In fig. 38, a chromatogram is shown of column fractions from an alumina column, these three fractions together make up fraction I in the gradient elution technique. The fast running component with an Rf similar to that of liquid paraffin presumably corresponds to the saturated hydrocarbon fraction expected in leaf waxes and the fraction running just behind it appears to be the steroid precursor squalene since Hemming, Morton and Pennock (1963) report that it runs in this position on TLC. The fluorescent component in fraction 1 is probably phytofluene and a non fluorescent compound sometimes observed running just ahead of it (not shown in fig. 38) may be phytoene. The major component of fraction 1, orange in colour, is carotene.

In fractions 2 and 3 in fig. 38, two groups of components can be seen, the faster running one being almost solely

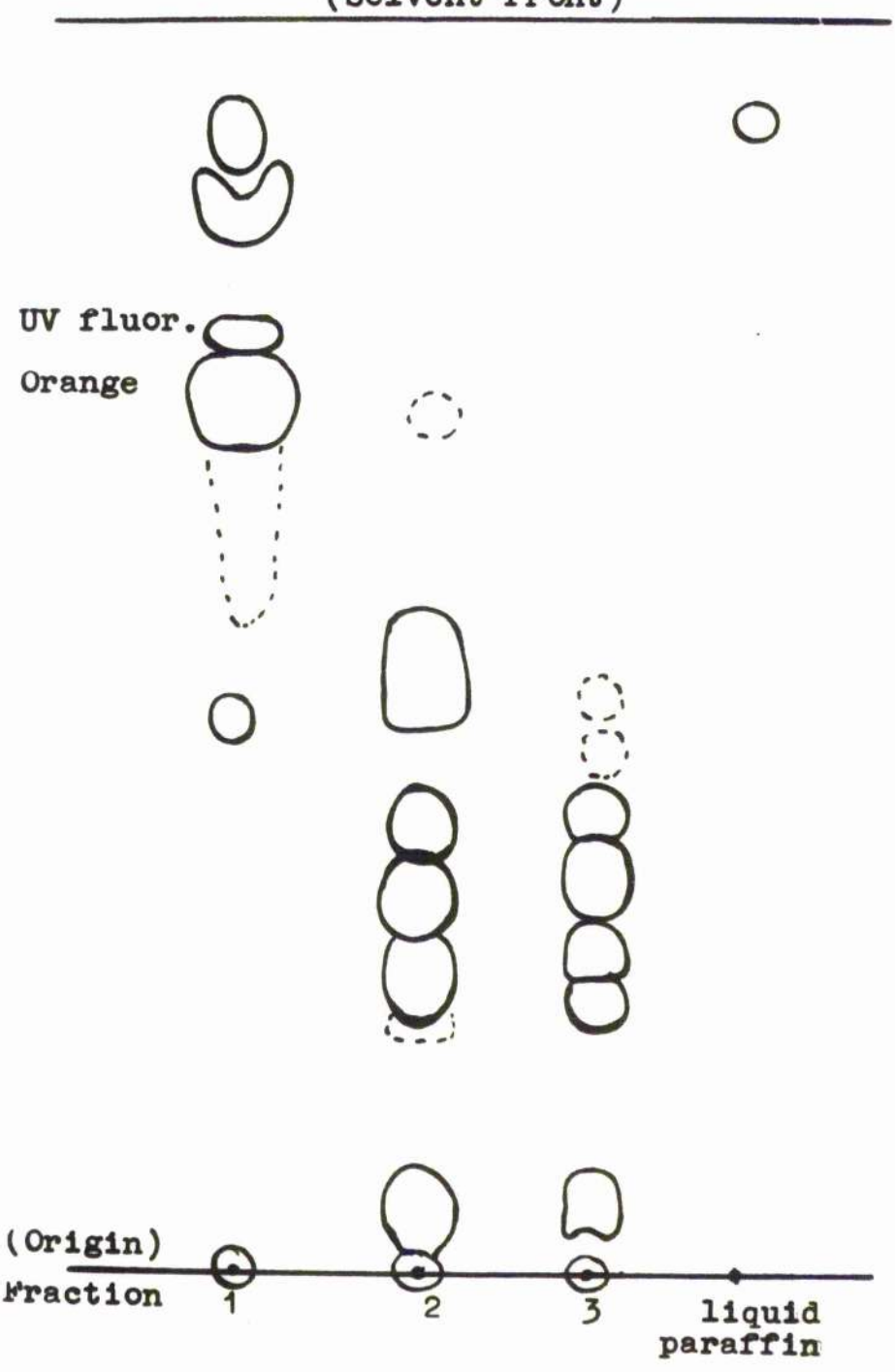
Fig. 38

TLC OF COLUMN FRACTIONS

Silica Gel G

Solvent: n-hexane (unlined tank)

Detection: 30% c.H_2SO_4/ethanol



present in fraction 2. The Liebermann-Burchard reaction showed the presence of sterol esters in the region of the chromatogram corresponding to the slower running group of components. The faster running group of compounds appears to correspond to those shown in fig. 34 which give a weak reaction with the neotetrazolium chloride reagent. These compounds appear to be wax-like but fairly unstable and easily saponified; the very slow running components in fig. 38 are breakdown products of some sort, possibly due to hydrolysis. The identity of this faster running component is uncertain since Holloway and Challen (1966) report that the alkyl esters found in waxes run behind the sterol esters on TLC, and long chain alkyl esters would also be more resistant to hydrolysis. The wax-like fractions usually found accompanying plastoquinone A are probably such alkyl esters.

The reaction of the neotetrazolium chloride reagent with the wax like fractions above is unexpected, since Lester and Ramasarma (1959) reported that this reagent was specific to quinones in the tissue extracts that they examined. This reagent has been modified in this present work however, and its specificity has obviously been altered. A positive reaction was also obtained with a fraction running below pigment number 1 on TLC and this, from examination of its acid-hydrolisis products, appears to be galactolipid.

In addition to the sterol esters described, the Liebermann-Burchard reaction showed the presence of another steroidal

fraction in leaf extracts and this runs below phaeophytin on TLC. This fraction was found to be free sterol and its anomalous behaviour on column chromatography has already been discussed. The speed of reaction of the Liebermann-Burchard reaction suggested that both "fast-acting" sterols (Δ^7 and $\Delta^{5,7}$) and "slow acting" sterols (Δ^5) were present.

The tocopherols in leaf extracts were detected on thin layer chromatograms by means of the reagents described in the Experimental Section (B 2), (viii) - (xi)). The Turnbulls' Blue reagent (ix) was found to be slightly more sensitive than the ferric-chloride/α,α'-dipyridyl reagent (viii) normally, and so the former was generally used throughout this work. The phosphomolybdic acid reagent (x) was useful in that heating the plate allowed it to be used as a general method of lipid detection. The diazo reagent (xi) was used as described to distinguish between the various tocopherols, and some other components were also revealed with this reagent. A white fluorescent band was observed running just ahead of phylloquinone in leaf extracts and this fraction gave a blue colouration with the diazo reagent. From its reaction, it is presumably phenolic in nature, and its Rf value would be consistant with it being a multiprenyl phenol of the type described by Rudney and Raman (1966) as an intermediate in ubiquinone biosynthesis. Rudney and Raman report that their multiprenyl phenol ran with an Rf value 2.33 times that of ubiquinone-10, whereas the diazo-positive compound found in

this work had an Rf value of 2.31 times that of ubiquinone-10 in the same system (benzene on silica gel). It would be of interest to see whether this compound corresponded to the decaprenyl phenol which would give rise to ubiquinone-10 or to a nonaprenyl phenol which could possibly be an intermediate in plastoquinone biosynthesis. Another compound has sometimes been observed in leaf extracts which also gives a blue colouration with the diazo reagent and is not a tocopherol. This compound is more polar than δ-tocopherol and runs in the same position as phaeophytin in TLC on silica gel. This compound gives a slow reaction with the neotetrazolium chloride reagent and may be another quinone precursor, such as the hydroxylated multiprenyl phenol described by Olson (1966).

α-tocopherol has been well authenticated in leaf extracts although Friend (1967-personal communication) has suggested that plastoquinol A may often be mistaken for α-tocopherol: both these compounds were found to run at the same Rf in this work. In an attempt to isolate the other tocopherols present in leaf extracts, the column fraction VI and the material eluted before fraction VII were collected from four separations and the extract strip loaded onto two 20 x 20cm 500μ silica gel plates. After development in chloroform in an S-chamber, three bands were found which gave a positive reaction with the Turnbulls Blue reagent. These three bands were eluted and samples rechromatographed together with authentic tocopherols. The separation achieved is shown in

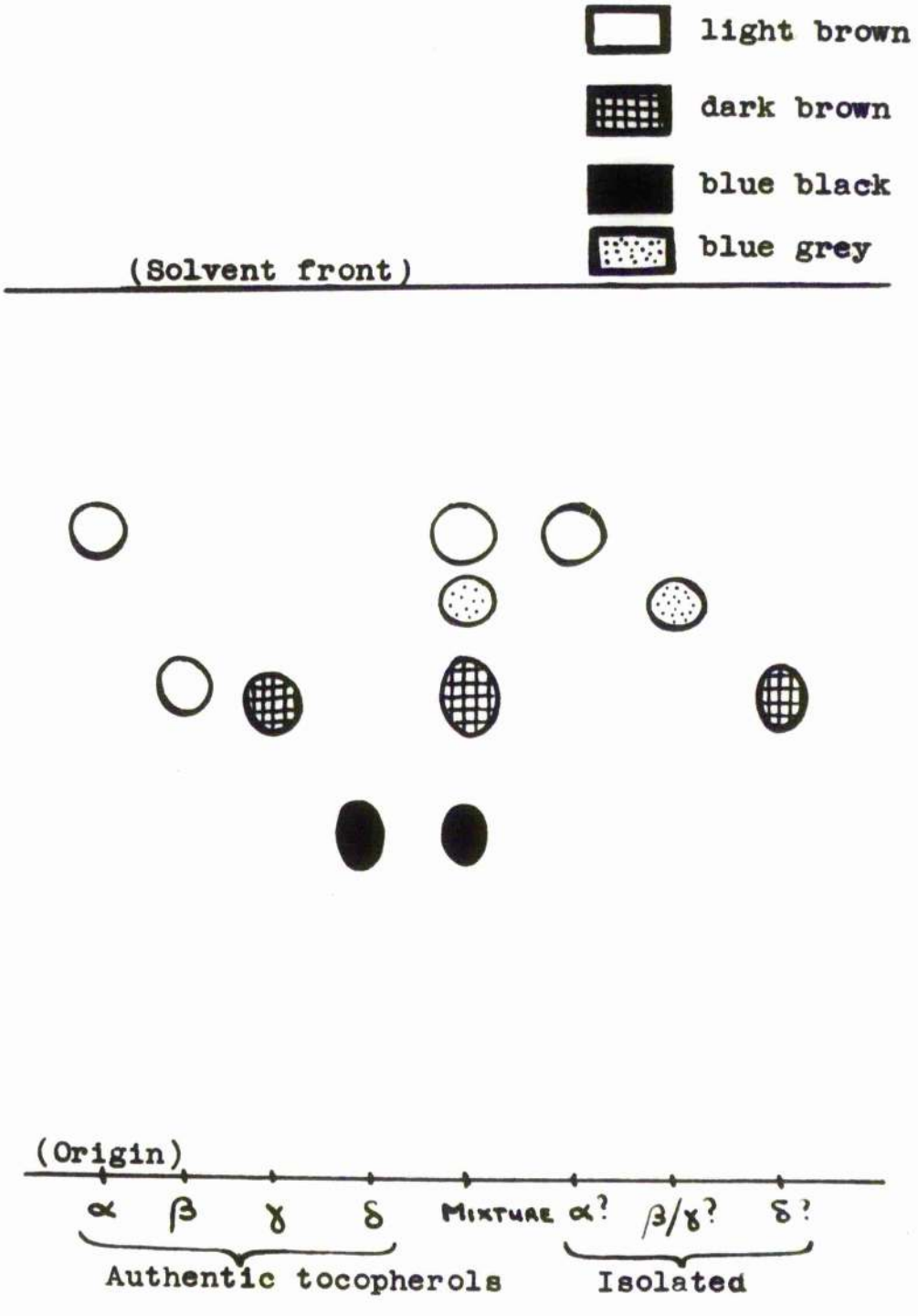
fig. 39 and, as can be seen, α-tocopherol is present. The fraction presumed to correspond to β and/or γ-tocopherol ran ahead of the authentic compounds and gave a blue-grey colouration with the diazo reagent. The leaf extract fraction originally thought to correspond to δ-tocopherol actually ran as a mixture of β and γ-tocopherols from its Rf value and the intensity of staining with the diazo reagent. The compound running between α- and β-tocopherol was purified, and ultra violet and infra red spectroscopy were performed on this compound. The results of these are described in section C and identify the compound as plastochromanol, a cyclic derivative of plastoquinone A, which has been reported by Whittle, Dunphy and Pennock (1965) and Dunphy, Whittle and Pennock (1966) Reinvestigation of the fraction running below β/γ-tocopherol on the original TLC plates did show the presence of a small amount of a compound with the chromatographic and staining properties of δ-tocopherol.

TLC OF PLANT CHROMANOLS

Silica Gel G

Solvent: chloroform (unlined tank)

Detection: diazo reagent-



RESULTS (B)

3) MODIFICATIONS OF THIN LAYER CHROMATOGRAPHY

a) Reversed-phase partition TLC

(i) Paraffin-impregnated silica gel

Reversed-phase techniques were used partly for purification of isolated compounds and partly for their characterization by comparison with authentic compounds. Paraffin-impregnated silica gel was used for both these purposes and fig. 40 shows a chromatogram of various partially purified column fractions, together with authentic quinones; the solvent was acetone/water (95:5) and the detection reagent was iodine vapour. As can be seen, the plastoquinone A fraction (III) cochromatographs with authentic plastoquinone-9 (PQ9). The ubiquinone fraction (VI) also cochromatographs with authentic ubiquinone-10 (Q-10), as does the phylloquinone fraction (II) with authentic phylloquinone (K). The fast running components in fractions VII and VIII are presumed (on other evidence) to be plastoquinones C and D, the latter together with α-tocopherolquinone. The slow running components in fraction V and the trace in fraction IIIA and IV appear to be plastoquinone B components and, although they normally appeared as elongated spots, double spots were sometimes observed in agreement with the findings of Griffiths, Wallwork and Pennock (1966). These plastoquinone B components are better resolved in systems containing less water, and the polar plastoquinones (C andD) and

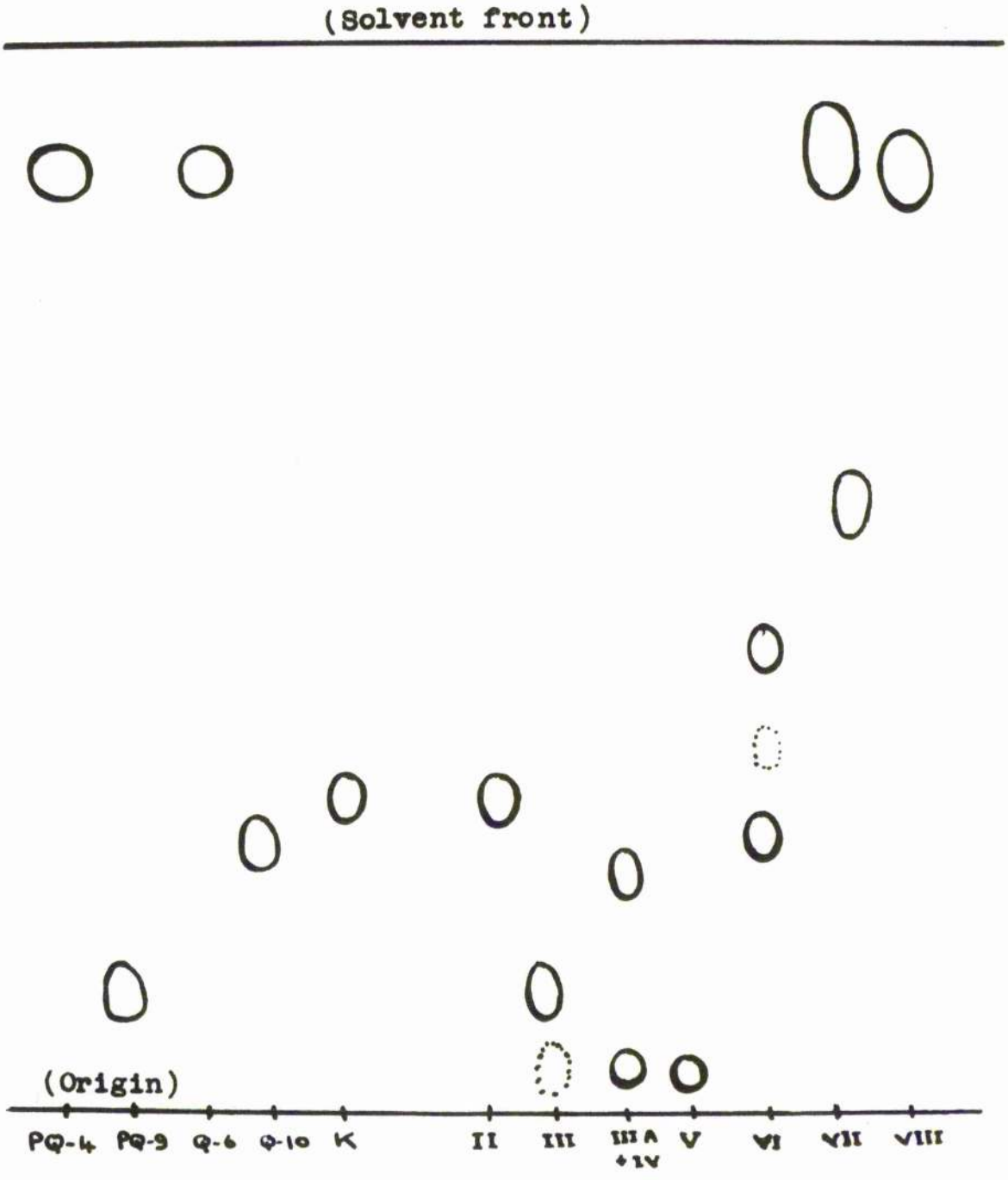
Fig. 40

TLC OF COLUMN FRACTIONS

Paraffin impregnated silica gel

Solvent: acetone/water (95:5)

Detection: iodine vapour



the tocopherolquinones are better resolved in acetone/ water mixtures containing 10-20% water.

The faster running component in the column fraction IIIA + IV was usually found, and from its chromatographic behaviour, it was thought that it might be a lower isoprenologue of plastoquinone A. Consden, Gordon and Martin (1944) and Martin (1949) postulated that the Rf value in partition chromatography was dependent only on the chemical structure of the compound. Martin (1949) also postulated that the addition of any group to a compound should change its Rf value by a specified degree, and Bate-Smith and Westall (1950) introduced the term R_M to cover Martin's additivity principle, where $R_M = \log_{10}(\frac{1}{Rf} - 1)$.

The sum of the effective R_M values for all the component groups in a molecule should theoretically give the R_M value for the compound itself and so, if the R_M value or $\triangle R_M$ for the isoprene unit could be determined, it should be possible to calculate whether or not the fast running component in fraction IIIA +IV in fig. 40 could be an isoprenologue for plastoquinone A and, if so, the length of its side chain.

Green and coworkers have examined the relationships between R_M values and chemical structure (Green and Marcinkiewicz, 1963; Marcinkiewicz, Green and McHale, 1963), and Green, Marcinkiewicz and McHale (1963) have shown that $\triangle R_M$ parameters can be applied to complex molecules such as the

tocopherols and ubiquinones using reversed-phase paper chromatography. Bark and Graham (1966a, 1966b) have shown that reversed-phase TLC gives equivalent or better results in the determination and correlation of R_M values for alkyl phenols, consequently it seemed feasible that R_M values may be applicable in this present work.

The above workers took great care to ensure the [illegible]-reproducibility of Rf and R_M values in their work, and since the present studies were devoted mainly to separation problems , similar controls were not applied; consequently Rf and R_M values were not very reproducible. A fixed relationship of Rf and hence R_M values did apply on any single plate and so, using the two ubiquinones and the two plastoquinones available, the ΔR_M for the isoprene unit could be calculated for any single plate. Table 6 shows the Rf and calculated R_M values for the ubiquinones and plastoquinones shown in fig. 40, and good agreement is shown between the ΔR_M for a single isoprene unit in the ubiquinones and in the plastoquinones (a difference of only 2%). The ΔR_M value for plastoquinone-9 and the unknown quinone in fraction IIIA +IV is 0.341, and this is consistent with it being the lower isoprenologue, plastoquinone-8. A trace of a compound with an R_M value close to that expected for plastoquinone-7 was also occasionally observed.

TABLE 6 Comparison of Rf and R_M values for the ubiquinones and plastoquinones shown in fig.40.

Compound	Rf	R_M*	$\triangle R_M$	$\triangle R_M$ for a single isoprene unit
Ubiquinone-6	0.88	-0.870	1.325	0.331
Ubiquinone-10	0.26	+0.455		
Plastoquinone-4	0.87	-0.824	1.690	0.338
Plastoquinone-9	0.12	+0.866		
Unknown in IIIA+IV	0.23	+0.525	-	-

* $R_M = \log_{10}(\frac{1}{Rf} - 1)$

(ii) Polyamide and polyethylene powder layers

Both polyamide and polyethylene powder layers gave satisfactory results with solvents of aqueous acetone similar to those used on paraffin-impregnated layers. On the whole, polyethylene powder layers gave sharper spots and a separation of various compounds on such a layer is shown in fig. 41. The solvent used was acetone/water (88:12) and the detection reagent was iodine followed by phosphomolybdic acid. These layers were useful for showing the presence of both α-tocopherol and ubiquinone-10 in column fraction VI. The plastoquinone B components on such layers were normally obscured by the carotenoid often found associated with such fractions.

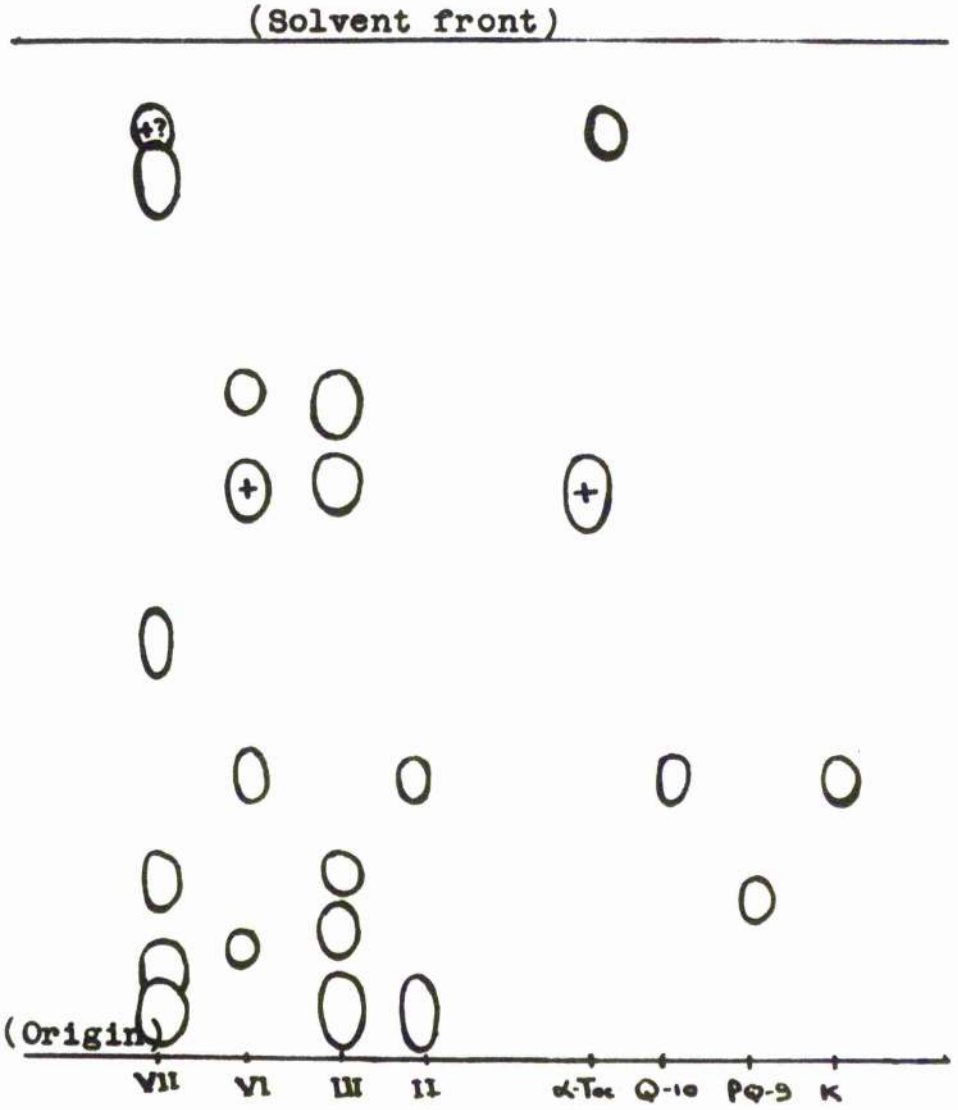
To obtain similar separations on polyamide layers, higher concentrations of water were necessary, and acetone/water (80:20) would be required with a polyamide layer to give an

TLC OF COLUMN FRACTIONS

Polyethylene powder

Solvent: acetone/water (88:12)

Detection: iodine vapour then phosphomolybdic acid (+ signifies blue colouration)



equivalent separation to that shown in fig. 41. A disadvantage of both these hydrophobic materials, as well as of the paraffin impregnated layers, was that destructive reagents such as concentrated sulphuric acid caused charring of the layer. In addition, polyethylene powder tended to fuse at the temperatures necessary for reaction.

Phylloquinone exhibited a specific reaction with polyamide layers in a pink colouration on drying the developed plate. This was presumably due to the basicity of the polyamide or a reaction with the amide groups which rendered this adsorbent unsatisfactory for purification or preparative purposes. Phylloquinone was also found to run with a somewhat higher Rf than ubiquinone-10 on such layers, in contrast to its behaviour on polyethylene powder and paraffin impregnated layers where there was very little difference.

b) Two dimensional TLC using adsorbent and reversed-phase techniques.

Comparatively poor results were obtained using paraffin impregnation for the second dimension and so most of this work was performed using silica gel for the first dimension and either polyamide or polyethylene powder in the second. In the case of polyethylene powder, it was found to be necessary to prewash the plate with benzene to remove a wax-like material, possibly a plasticizer, from the layer. The solvents used on such layers were benzene in the first dimension and the solvents described in section a)(ii) above for the second dimension. The plate must be thoroughly

dried with a jet of nitrogen after the first development to remove all trace of benzene.

Fig. 42 shows a composite chromatogram of a whole leaf extract washed with 80% and 90% aqueous methanol (as in the gradient elution chromatography of the quinones) to remove the polar lipids which distort the separation. The solvent was benzene in the first dimension and acetone/water (88:12) in the second, and the identifications shown in table 7 are based on the reaction with neotetrazolium chloride, phosphomolybdic acid or iodine vapour, together with the appearance of the spots under ultra violet light and information from other techniques. Compound 8 in fig. 42 is identified as sterol on the basis of its Liebermann-Burchard reaction but no colouration was seen with the fraction presumed to contain sterol ester.

Fig. 42

Two-dimensional adsorbent/reversed phase TLC

Solvent 1 - benzene
2 - acetone/water (88:12) } (S-chamber)

▭ : positive reaction with the neotetrazolium chloride reagent

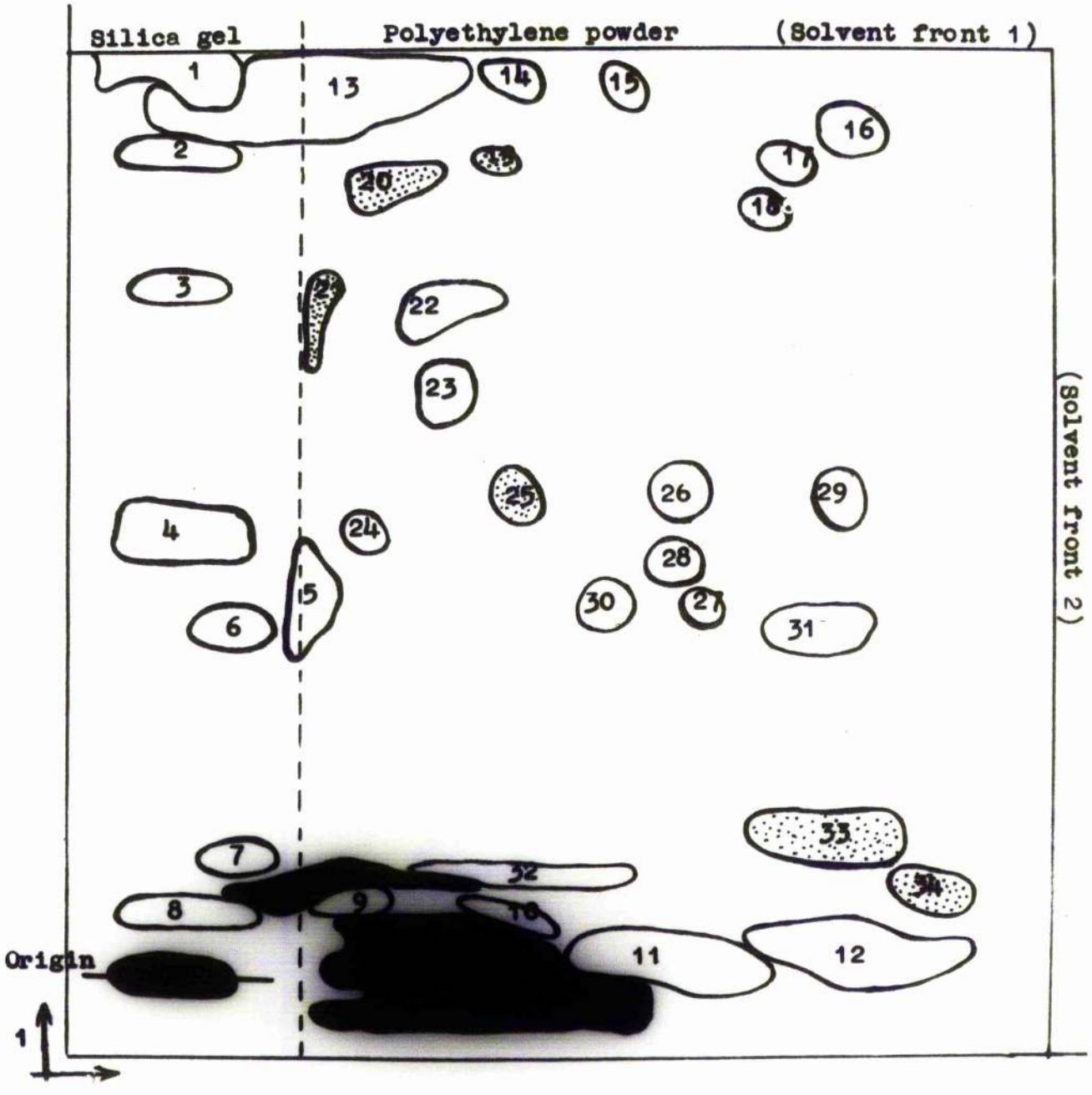


TABLE 7 Key to figure 42

Number	Appearance	Reaction with iodine vapour	Reaction with neotet. chloride	Identification
1	-	+	-	Hydrocarbons including squalene
2	-	+	-	Wax + sterol esters
3	-	+	-	Alkyl ester waxes
4,6	-	+	-	Triglycerides?
7	-	+	-	-
8	-	+	-	Sterol
9-12	Yellow	-	-	Xanthophylls
13	Orange	-	-	Carotene
14,15	UV fluorescent	-	-	Phytofluene +?
16-18	-	+	-	-
19	UV absorbent	+	+	Phylloquinone
20	do	+	+	Plastoquinone A
21	do	+	+	Plastoquinone B
22	-	+	-	-
23	UV fluorescent	-	-	-
24	-	+	-	-
25	UV absorbent	+	+	Ubiquinone-10
26	do	+	-	α-tocopherol
27	do	+	-	β/γ-tocopherol
28-31	-	+	-	Probably isoprenoid alcohols
32	-	+	-	-
33	UV absorbent	+	+	Plastoquinones C+D
34	do	+	+	α-tocopherolquinone

As can be seen from table 7, there are a number of unidentified lipid components in fig. 42, some of which fluoresce. A number of neutral lipid fractions do not move at all in the second dimension, and are left in the silica gel corridor. The group of lipids close to α-tocopherol are presumed to be isoprenoid alcohols reported by Wellburn and Hemming (1966a, 1966b), Hemming (1967) and Stone, Wellburn, Hemming and Pennock (1967). Authentic phytol runs in the region of spot 31 in this system and the longer chain isoprenoid alcohols would presumably run with a slightly lower Rf.

Attempts were also made to obtain two dimensional separations on mixtures of silica gel and polyethylene powder and some success was achieved using the same solvents as above, although the spots produced tended to be more diffuse. Also, as in the case of the polyethylene powder/silica gel layers above, activation of the silica gel had to be performed by thorough drying below 100°C to avoid fusion of the particles of the layer.

c) Two dimensional adsorbent TLC

Very little work was performed on this modification of TLC, although results were obtained which suggested the presence of a green chlorophyll-like substance which is less polar than the other chlorophylls i.e. it runs ahead of them in normal adsorption systems. This is probably the same compound found by Booth (1962), although it does

not appear to have been reported by any other workers.

d) Multiple development

Some separations were achieved by multiple development of TLC plates, but due to the instability of the qinones, this was not considered to be very reliable for preparative techniques and better results were obtained by the combination of some of the other separation techniques described.

e) Continuous TLC

Continuous TLC was performed as described in the Experimental Section, and separation of the carotene fraction of leaf extracts on silica gel was able to show the presence of α-carotene. By a comparison of the intensity of colour of the α- and β-carotene fractions it was estimated that the carotenes of the leaf consisted of about 5% α-carotene and 95% β-carotene.

Continuous TLC of leaf extracts on alumina layers was analagous to column chromatography and, as such, separations of phylloquinone and plastoquinone A were fairly easily obtained with solvents of 2% and 4% dichloroethane/n-heptane.

RESULTS (B)

4) PAPER CHROMATOGRAPHY

a) Silicic acid impregnated paper

Of the solvents tried on the Whatman SG81 paper used, only two produced satisfactory separations of the carotenoids present: chloroform and hexane/ether (30:70). Hexane/acetone (90:10) gave fairly good separations of some of the less polar pigments and possibly some of the quinones, although these were not tested for. Figs. 43 (a) and 43(b) show the separations of normal leaf extract (i.e. not saponified) achieved with hexane/ether (30:70) and hexane/acetone (90:10) respectively. In fig. 43(a) the presence of two carotenoid bands between lutien and violaxanthin can be seen, corresponding to fractions III and IV found on some TLC plates. In fig. 43(b) the chlorophylls and more polar xanthophylls are not well separated, but a yellow-orange xanthophyll is clearly visible at Rf 0.27, presumably corresponding to fraction VIII on TLC plates. The pale greenish yellow band at Rf 0.5 appeared pink under ultra-violet light (carotenoids normally appear grey) and so may possibly correspond to the "non-polar chlorophyll" found in setion 3) and by Booth (1962).

b) Alumina impregnated papers

Lichtenthaler (1964) and Lichtenthaler and Calvin (1964) have reported separations of the quinones and fat-soluble pigments of leaf extracts using a Schleicher and Schüll alumina

Fig. 43

CHROMATOGRAMS OF LEAF EXTRACT

Silicic acid impregnated paper

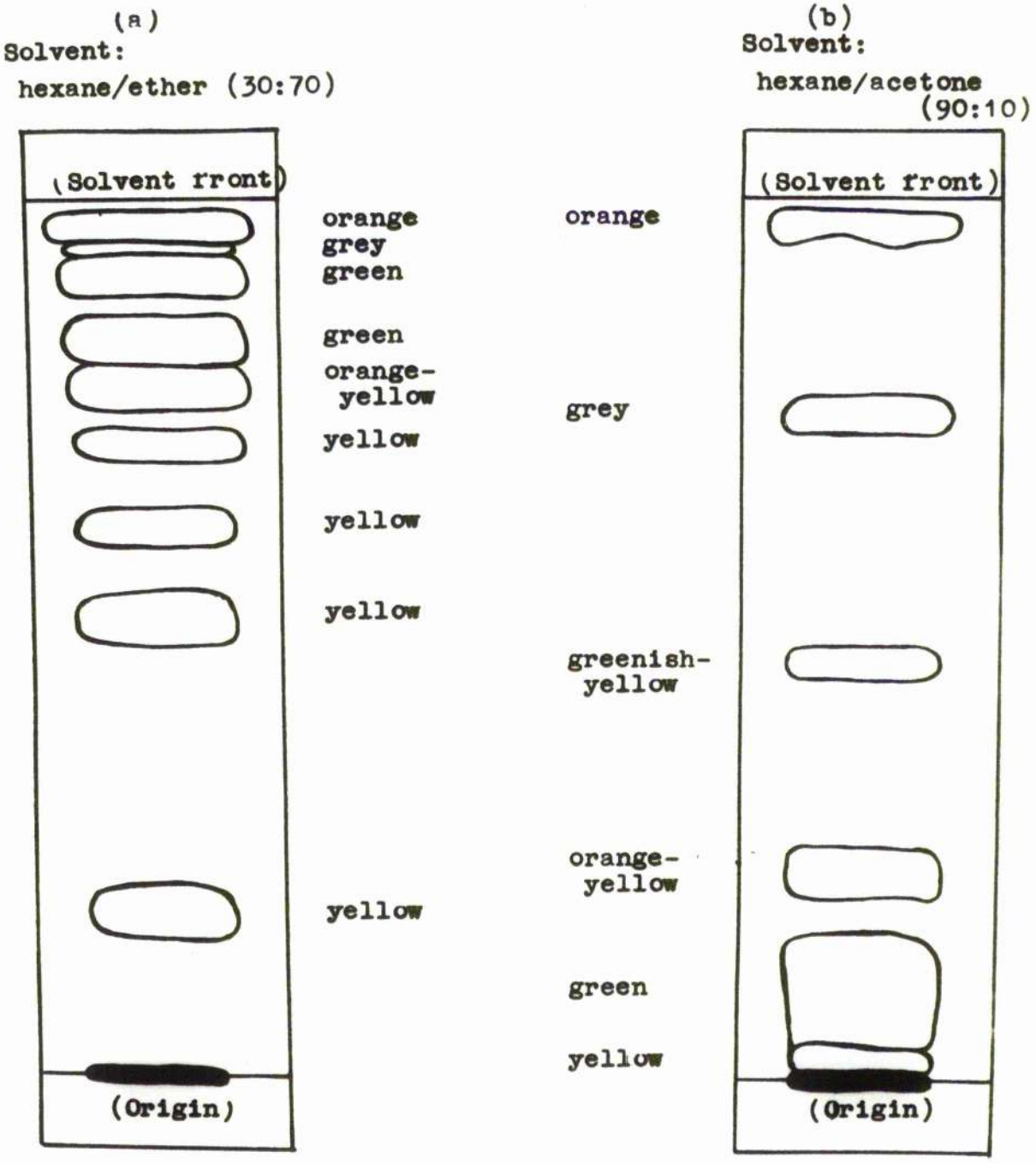


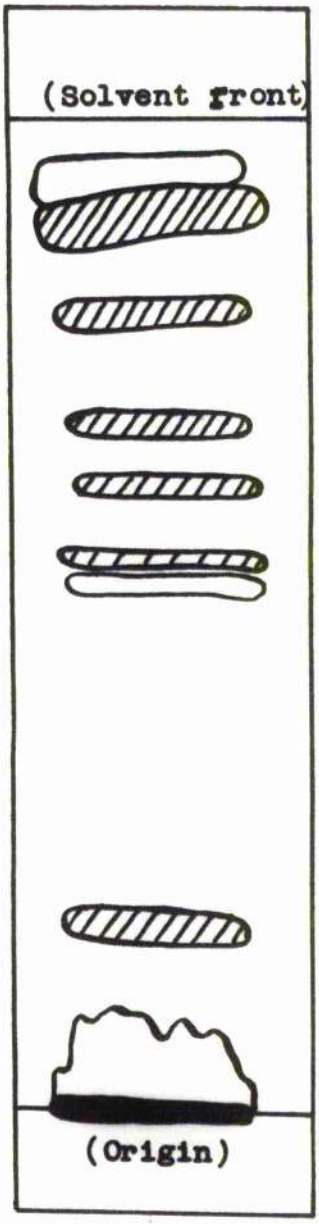
Fig. 44

CHROMATOGRAM OF LEAF EXTRACT

Alumina impregnated paper

Solvent: benzene

Positive with the neotetrazolium chloride reagent



Tentative identirication:

-carotene

Phylloquinone

Plastoquinone A

Coenzyme Q_{10}

Plastoquinone C

Plastoquinone D?
Phaeophytin

-tocopherolquinone

Chlorophylls and xanthophylls

impregnated paper with solvents of cyclohexane and benzene (separately and as mixtures of the two) and similar results have been achieved in this work. A separation of leaf extract on Whatman AH81, using benzene as solvent, is shown in fig. 44 and the identification is based mainly on the comparable chromatograms of Lichtenthaler (1964). The detection of the quinones was achieved by dipping the chromatogram in the neotetrazolium chloride reagent according to Lester and Ramasarma.(1959).

c) Silicone fluid impregnated paper (method of Lester and Ramasarma, 1959).

Several chromatograms were run using these silicone fluid impregnated papers and were mainly used for the reversed-phase characterization of column and TLC fractions, by comparison with the authentic compounds and literature reports.

The first chromatogram was run on paper impregnated with MS.555 silicone fluid, but was unsuccessful due to the solubility of this silicone fluid in the alcoholic developing solvent used: n-propanol /water (4:1). Later chromatograms, one of which is shown in fig. 45, were run on paper impregnated with MS.550 and equilibrated with the solvent for one hour before development. The results are fairly satisfactory and the slight streaking observed could probably be prevented by a longer period of equilibration.

A comparison of the Rf values obtained with those given by Kegel, Henninger and Crane, 1962 ("ref.1") and Crane and Dilley/1963("ref.2") is presented in table 8 below.

CHROMATOGRAM OF LEAF EXTRACT

Silicone fluid impregnated paper

Solvent: n-propanol/water (4:1)

Detection with the neotetrazolium chloride reagent according to Lester and Ramasarma (1959).

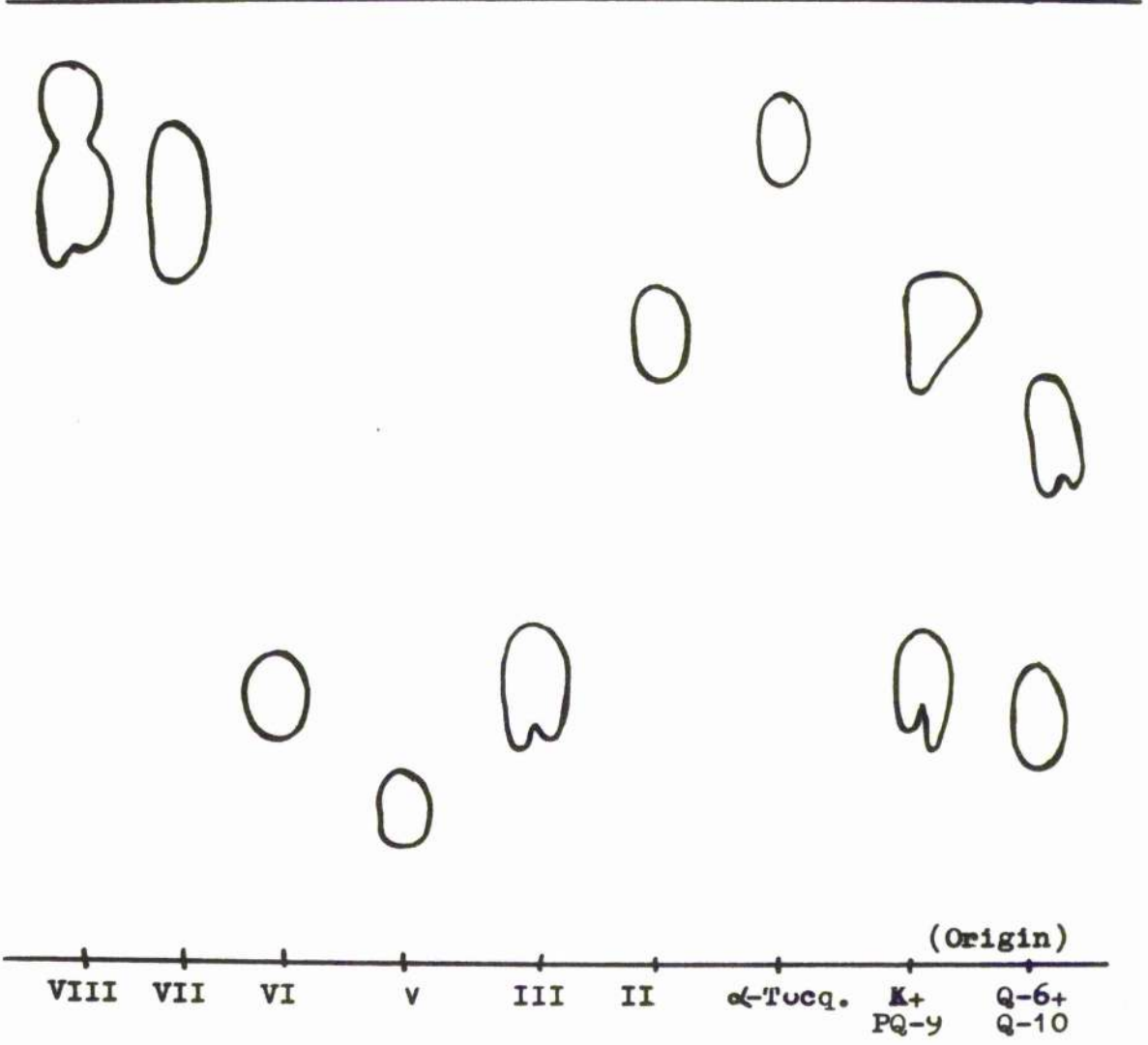


TABLE 8 Analysis of figure 45

Column fraction number	Identification	Rf value in the fig. 45	Rf values in the literature "ref.1"	"ref.2"
-	Authentic ubiquinone-6	0.56	-	0.54
-	Authentic ubiquinone-10	0.26	0.29	0.27
-	Authentic phylloquinone	0.68	0.63	0.59
-	Authentic plastoquinone-9	0.30	0.27	0.25
-	Authentic α-tocopherol-quinone	0.86	-	-
II	Phylloquinone	0.66	0.63	0.59
III	Plastoquinone A	0.30	0.27	0.25
V	Plastoquinone B	0.17	0.13	0.18
VI	Ubiquinone-10	0.28	0.29	0.27
VII	Plastoquinone C	0.80	0.75	0.81
VIII	Plastoquinone D? + α-tocopherolquinone	0.81? 0.89?	- -	- -

RESULTS (C)

SPECTROPHOTOMETRY

1) ESTIMATION OF CHLOROPHYLLS

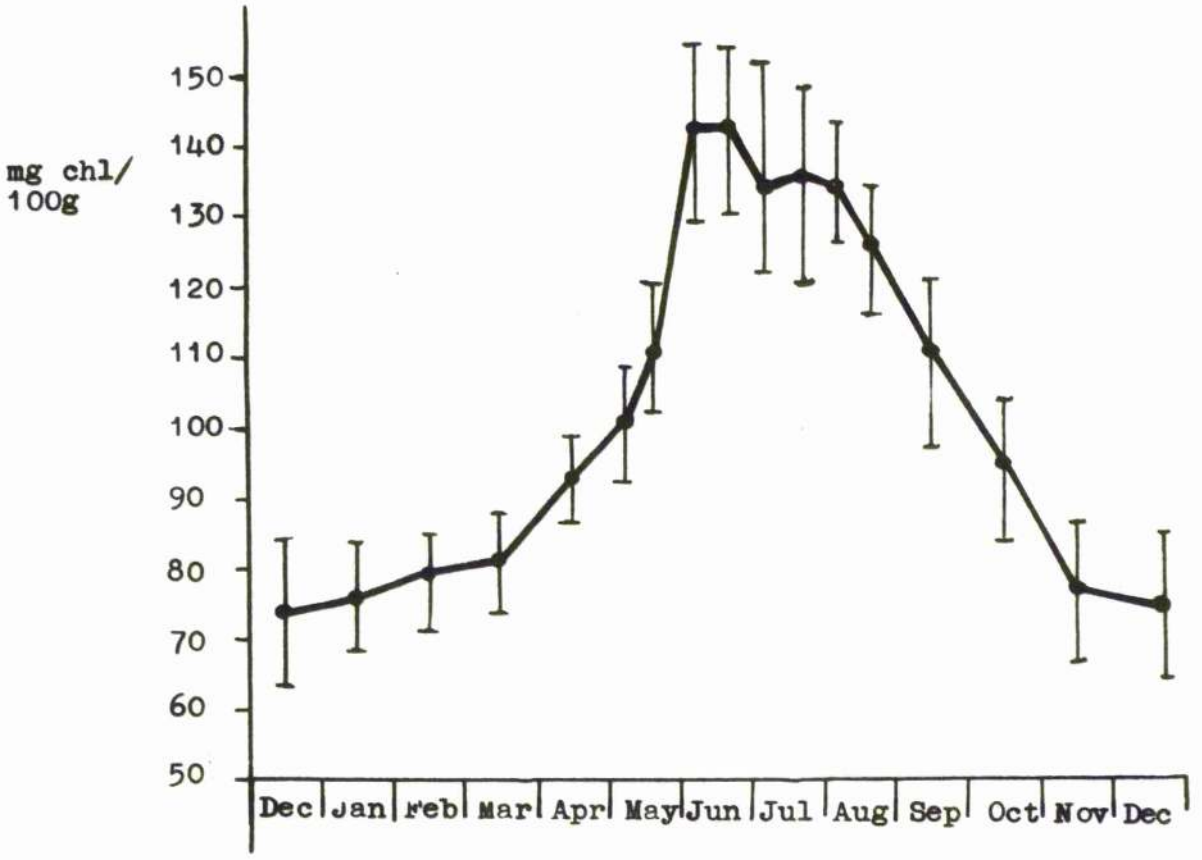
The chlorophylls in the leaf extracts were estimated spectrophotometrically by the method of Comar and Zscheile (1942), as described in the Experimental Section. There was a variation in total chlorophyll from 65.7 to 152 mg/100g fresh weight of leaf material; Comar and Zscheile (1942) found values ranging from 125mg/100g for tobacco to 297mg/100g for wheat. The lower values in this work were found in plants under winter conditions and the variation in the total chlorophyll content of spinach beet leaves throughout the year is shown in fig. 46. As can be seen, there is an increase in total chlorophyll from the end of March onwards, with a rapid rise at the end of May and a slow fall during the autumn.

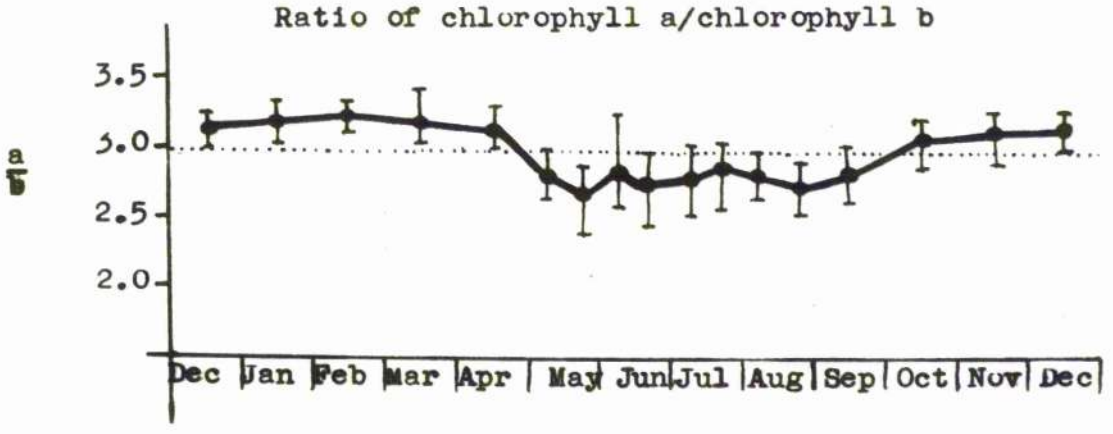
The values of chlorophylls/chlorophyll b found, vary between 2.36 and 3.42, with a mean of 2.97 as compared with the average value of 2.38 found by Lichtenthaler and Calvin (1964) for spinach chloroplasts (on a limited number of estimations). Thirkell (1961) found the ratio chlorophyll a/chlorophyll b to vary mainly between 1.29/1 and 5.50/1 (with two exceptional values of 7.92/1 and 9.79/1). Willstätter and Stoll (1913) reported a range of chlorophyll a/chlorophyll b ratios of from 2.05 to 3.52 for various plants.

The concentration of chlorophyll a found, varies from

Fig. 46

Seasonal variation of chlorophylls in the leaves of Spinach beet





70.5% to 77.5% of the total chlorophyll, whereas Comar and Zscheile (1942) report values of 68.1% to 77.2% for various plants.

As can be seen in fig.46 the lower values of the chlorophyll a/chlorophyll b ratio were generally obtained during the summer when the total chlorophyll concentration was highest. This may represent a preferential increase in chlorophyll, or alternatively, a preferential photochemical destruction of chlorophyll a at higher light intensities.

RESULTS (C)

2) IDENTIFICATION AND ESTIMATION OF CAROTENOIDS

The absorption spectra of the carotenoids were determined as described in the Experimental Section, and sample spectra obtained for most of the pigemnts found are shown in figs. 47-54, together with their acid conversion products where applicable. The absorption maxima found are represented on the figures and also, in tabular form, in tables 9 and 10, together with the absorption maxima reported in the literature for these carotenoids. As can be seen, there is a great deal of variation, even between different tables in the same paper (see Bickoff, Livingston, Bailey and Thompson, 1954); this is probably due, in most cases, to the variation in the instruments used.

The actual shape of the curve of an absorption spectrum is often a better means of recognition of individual carotenoids and an indication of this is given by the "relative optical density" values given in table 11. These values are a measure of the optical densities at the absorption maxima with that at the wavelength of maximum absorption arbitrarily being given the value of 1.0. The values obtained are generally in fairly good agreement with those found by other workers.

Figs. 47-49 also show the absorption spectra of the acid conversion products of these xanthophylls, showing a decrease in wavelength of the major absorption maxima of

FIG. 47

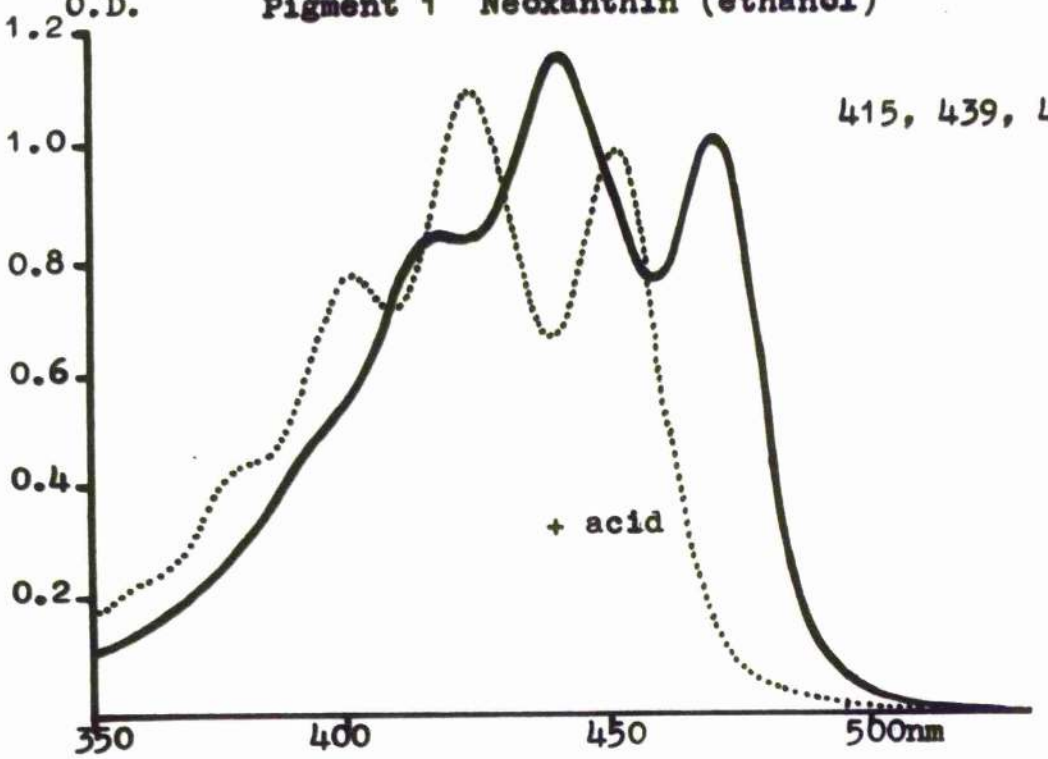

O.D.
Pigment 1 Neoxanthin (ethanol)
415, 439, 468
+ acid
1.2
1.0
0.8
0.6
0.4
0.2
350
400
450
500nm

FIG. 48

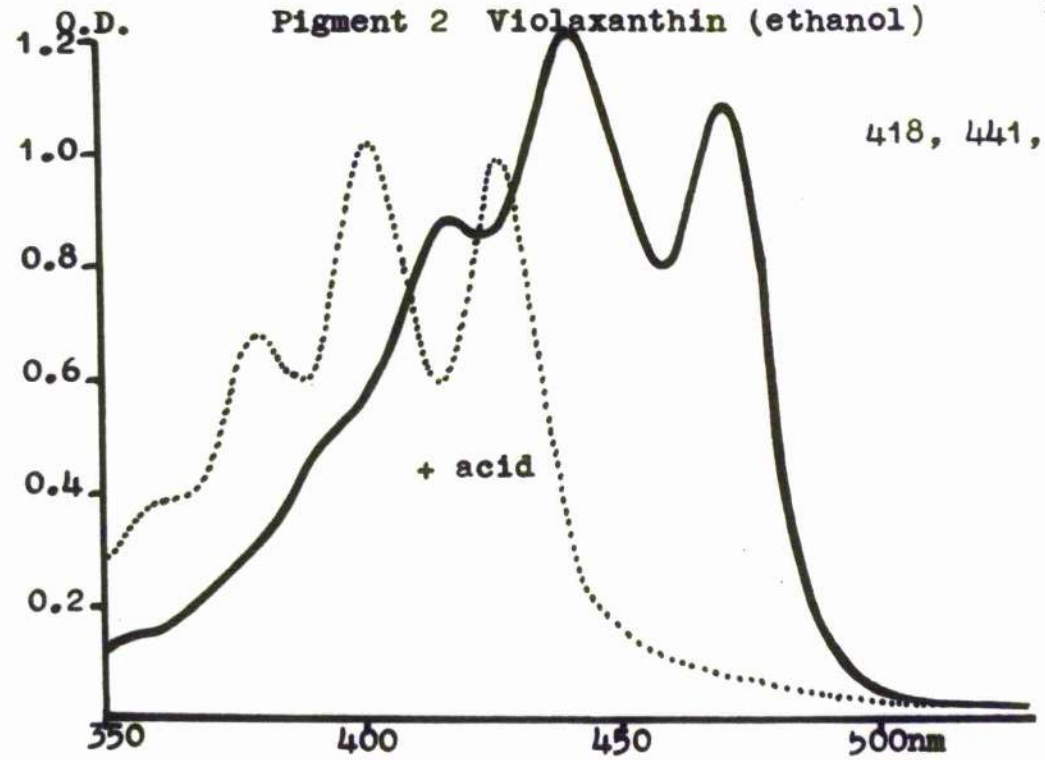
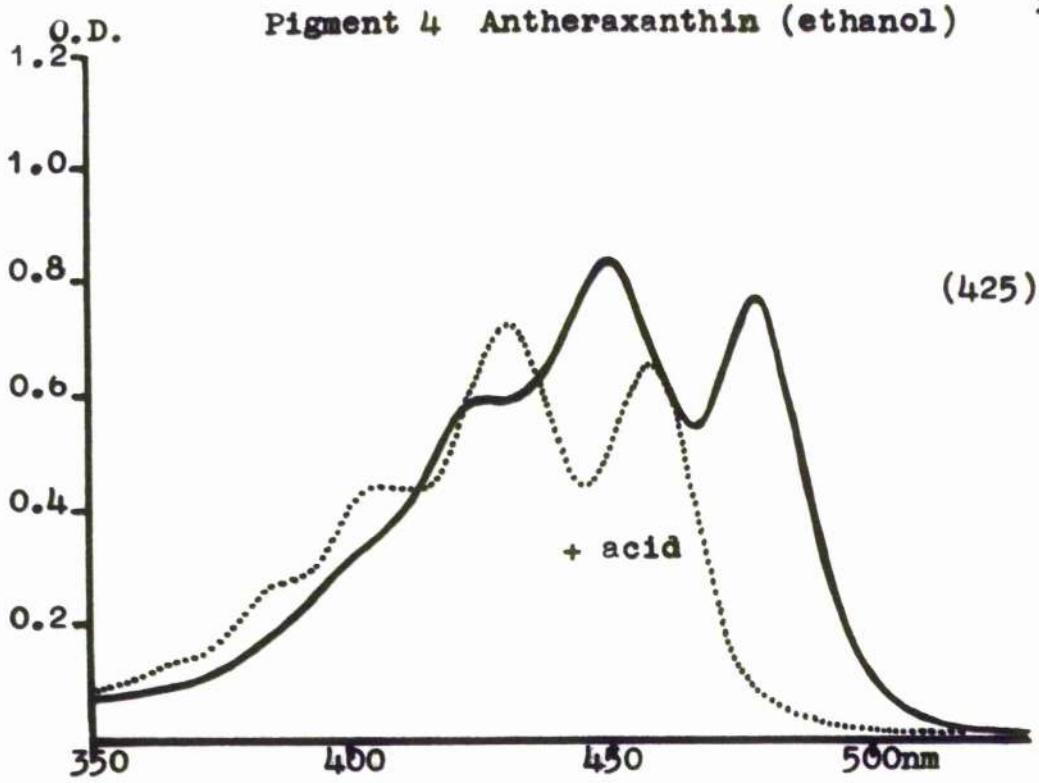
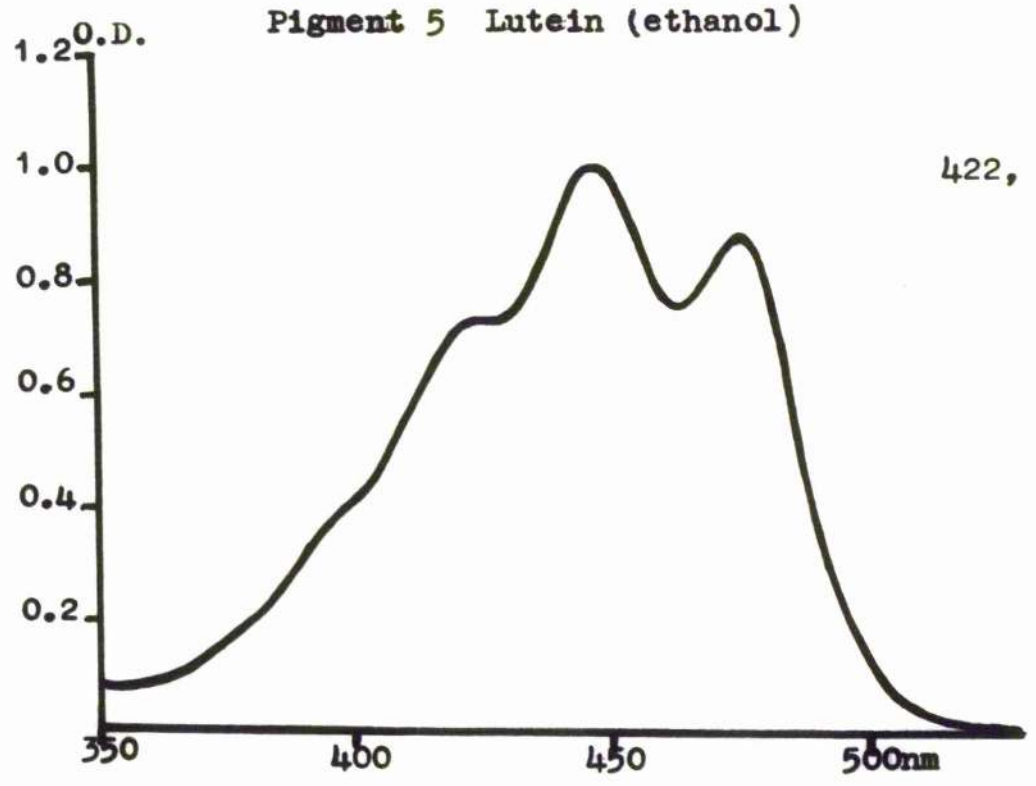

O.D.
Pigment 2 Violaxanthin (ethanol)
418, 441, 470
+ acid
1.2
1.0
0.8
0.6
0.4
0.2
350
400
450
500nm

FIG. 49



(425), 448, 477

FIG. 50



422, 446, 475

FIG. 51

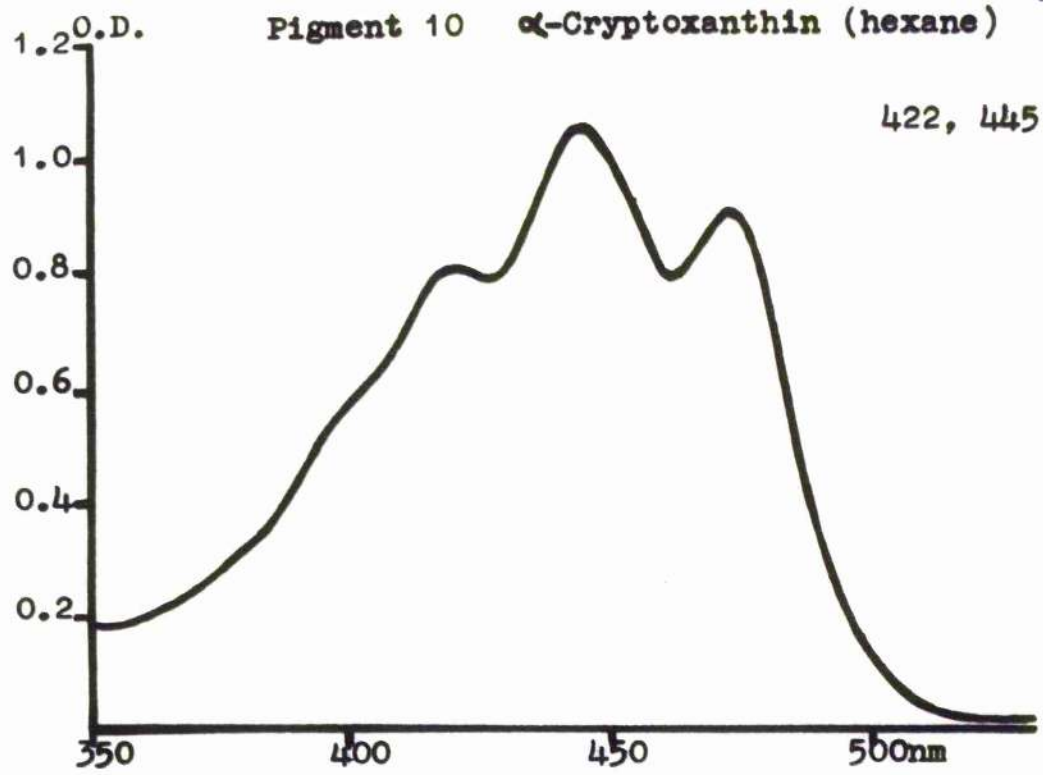

O.D.
Pigment 10 α-Cryptoxanthin (hexane)
422, 445, 474
1.2
1.0
0.8
0.6
0.4
0.2
350
400
450
500nm

FIG. 52

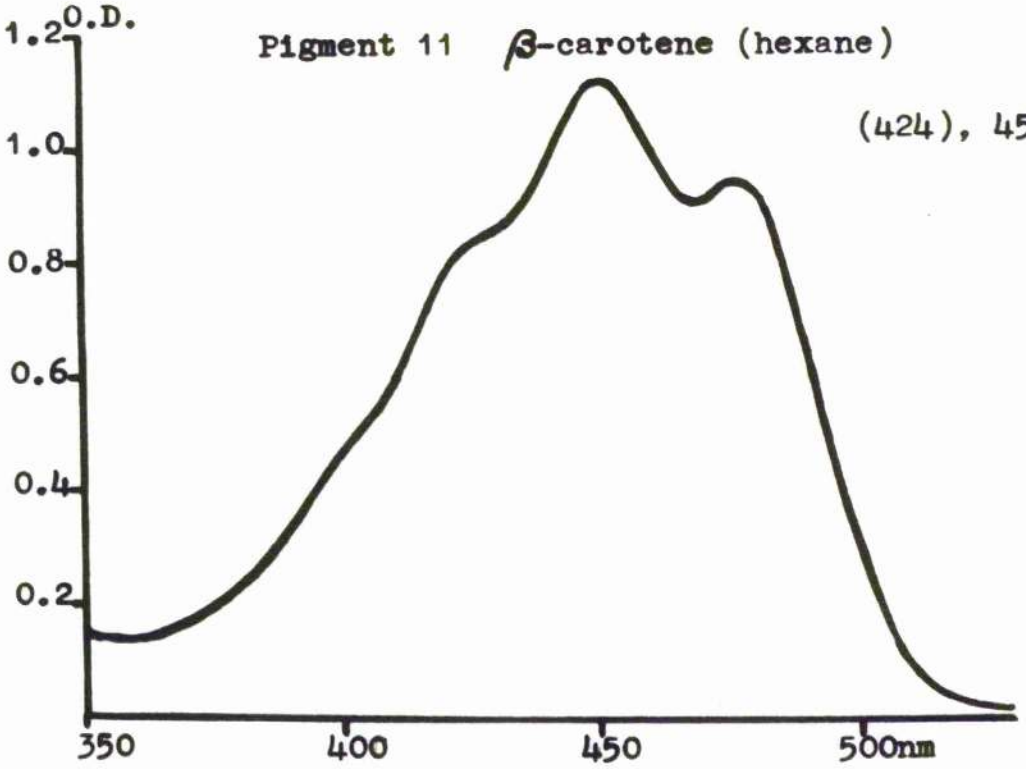
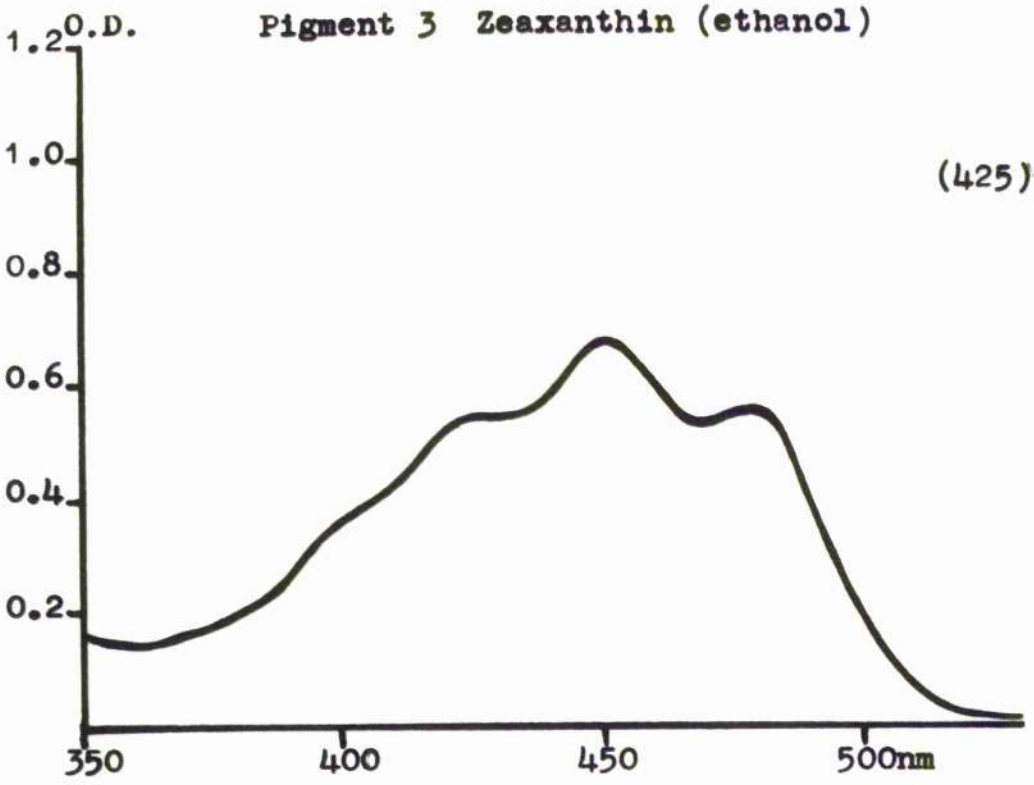
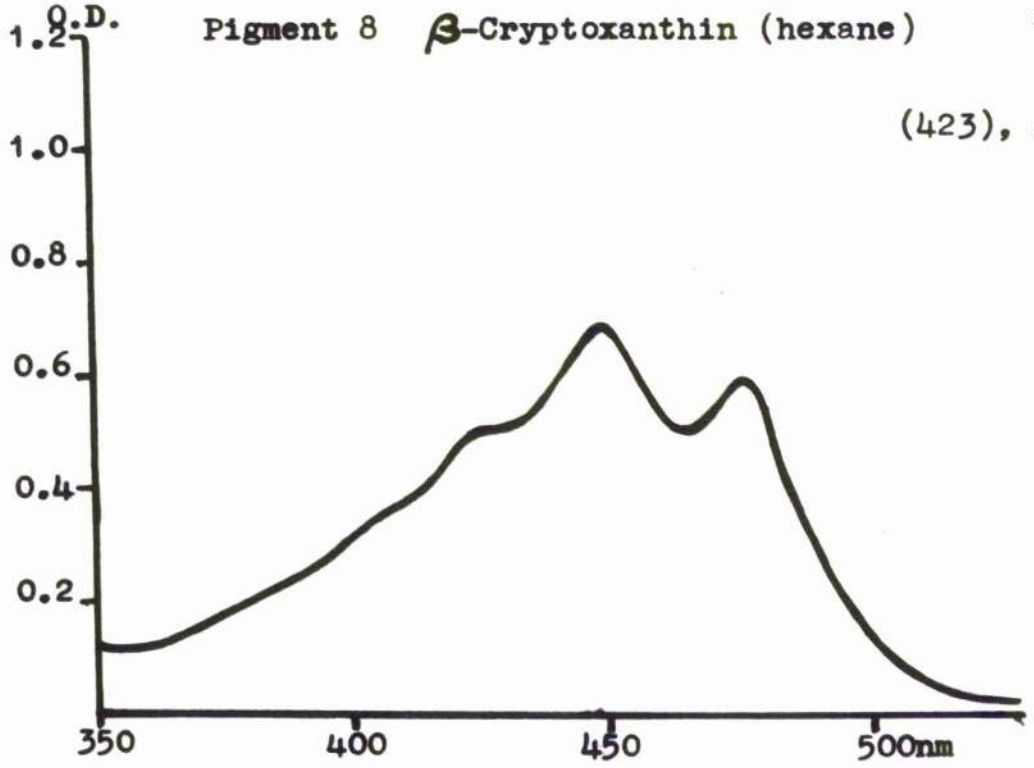

O.D.
Pigment 11 β-carotene (hexane)
(424), 450, 477
1.2
1.0
0.8
0.6
0.4
0.2
350
400
450
500nm

FIG. 53



(425), 452, 478

FIG. 54



(423), 448, 476

about 18-20nm for a single epoxide group and about 38nm for the two epoxide groups of violaxanthin. Pigment IA appeared to correspond to the furanoxide formed by acid treatment of neoxanthin and so, whether it is an artefact of isolation or not is uncertain. None of the minor pigments sometimes found between pigments 10 and 11 showed any evidence for the presence of an epoxide group.

Pigment 3 appears to correspond to zeaxanthin, but it was only occasionally present and the absorption spectrum shown in fig. 53 was obtained during the course of the etiolation experiments, the results of which are described later. Good spectra were never obtained for pigment 8, but the spectrum shown in fig. 54, together with others obtained, suggests that it is probably β-cryptoxanthin. Difficulty was sometimes experienced with this and other minor fractions due to the presence of traces of chlorophyll breakdown products, including phaeophytin, which had absorption maxima around 410nm. These often gave the appearance of carotenoid absorption spectra with excessively high short wavelength maxima. This effect could be checked for by the presence of a chlorophyll-type absorption maximum between 640 and 680nm. except in cases of very slight contamination. The absorption of such chlorophyll derivatives at 640-680nm was normally about half of that around 410nm.

Some evidence was also obtained of minor carotenoids other than those described. One of these with absorption maxima of 402, 426, 452 (in n-hexane) resembles citroxanthin

or mutatochrome as described by Booth (1962) and evidence has also been obtained of traces of a carotenoid with absorption maxima of 428, 456, 486nm (in n-hexane) which may be δ-carotene. Whereas α-cryptoxanthin was present at concentrations ranging from 25-70µg/100g fresh weight of leaf (representing about 0.4% of the total carotenoids), β-cryptoxanthin was less than 30µg and the other minor carotenoids found, less than 5µg/100g fresh weight of leaf. A sample of leaf extract from lucerne grown in Israel (provided by British Glues and Chemicals Ltd.) was found to contain 22% of its total carotenoids as α-cryptoxanthin, but the reason for this is unknown. No evidence for the distinctive short wavelength peaks of cis isomers was found in any fresh leaf extracts examined in this work.

The wavelengths of maximum absorption of each of the major xanthophyll components were determined (in ethanol) as: neoxanthin, 441nm; violaxanthin, 441nm; antheraxanthin, 448nm; lutein, 445.5nm. These wavelengths were used for the quantitative determination of the carotenoids in leaf extracts, as described in the Experimental Section. The results of some of these quantitative determinations are recorded in table 12 as mg of pigment/100g of fresh leaf material and as mg of pigment/100mg of total chlorophyll. The concentration of pigment 3 is calculated assuming it to be antheraxanthin (see Yamamoto, Nakayama and Chichester, 1962) and where it is not recorded it is included in the

lutein fraction.

The variation in total carotenoid is from 10.33 to 15.88 mg /100g of fresh leaf or 13.84 to 24.17mg /100mg of chlorophyll. This is unexpectedly more constant as regards fresh weight than when correlated with the chlorophyll content. The individual carotenoids present show a similar range of variation apart from antheraxanthin (?) on which, however, there are too few results to draw any conclusions, although antheraxanthin would be expected to show the greatest variation (from the results of Yamamoto, Nakayama and Chichester, 1962). The average values from table 12 are shown in table 14 in comparison with the results of other workers.

TABLE 9 Absorption maxima of carotenoids in n-hexane

Pigment	Absorption Maxima		
	Found	Literature	Ref.
1 Neoxanthin	412,435,463	412, 436, 466	2
		414, 437, 465	2
		- , 437, 466	3
		- , 437, 466	5
2 Violaxanthin	416.5,439,469	417, 441, 471	2
		- , 443, 472	3
4 Antheraxanthin	418,442,466	-	
5 Lutein	421.5,445,472	422, 447, 476	2
		420, 445, 474	2
		420, 447, 477	3
8 β-cryptoxanthin	(423),448,476	- , 449, 477	1
		- , 447, 473	2
		425, 451, 483	3
		(423-4),446,473-44	
		(424),452,485	6
10 α-cryptoxanthin	422,445,474	422,445.5,474*	1
		421-2,444-5,473*	2
		421, 446, 475	6
11 β-carotene	(424),450,477	- , 450, 478	1
		(425),451,482	3
		(425),448,474	4

* given for "monohydroxy α-carotene-like pigments".

See page 172 for the literature references.

TABLE 10 Absorption maxima of carotenoids in ethanol

Pigment	Epoxide Groups	Absorption Maxima Found	Literature	Ref.
1 Neoxanthin	1	415,439,468	413.5,438,467	1
			413-4,437-8,466	4
2 Violaxanthin	2	418,441,470	418,443,471	1
			420,441,471	3
3 Zeaxanthin	0	(425),452,478	- ,450,479	1
			423.5,451,483	3
4 Antheraxanthin	1	(425),448,477	424,447,477	4
			424,447,477	7
5 Lutein	0	422,446,475	424,446,476	1
			420,446.5,476	3
10 α-cryptoxanthin	0	422,447,476	422,448,478	6

References for tables 9 and 10:

1) Moster, Quackenbush and Porter (1952)

2) Bickoff, Livingston, Bailey and Thompson (1954)

3) Goodwin (1955)

4) Krinsky and Goldsmith (1960)

5) Goldsmith and Krinsky (1960)

6) Cholnoky, Szabolcs and Nagy (1958)

7) Davies (1961) quoted in Davies (1965)

TABLE 11 Relative optical densities of carotenoids

Pigment	Solvent	Relative optical densities Found	Literature	Ref
1 Neoxanthin	hexane	.682,1,.890	.655,1,.935	2
			.700,1,.825	2
	ethanol	.730,1,.887	.670,1,.965	1
2 Violaxanthin	hexane	.705,1,.915	.690,1,.950	2
	ethanol	.730,1,.910	.685,1,.960	1
3 Zeaxanthin	ethanol	.785,1,.815	- ,1,.890	1
4 Antheraxanthin	ethanol	.725,1,.915	.690,1,.910	3
5 Lutein	hexane	.670,1,.880	.680,1,.895	2
			.685,1,.890	2
	ethanol	.720,1,.885	.685,1,.920	1
8 β-cryptoxanthin	hexane	.715,1,.870	- ,1,.860	1
			- ,1,.820	2
10 α-cryptoxanthin	hexane	.770,1,.860	.695,1,.900*	1
			.705,1,.870*	2
			.680,1,.910	4
	ethanol	.730,1,.880	.672,1,.925	4
11 β-carotene	hexane	.720,1,.875	- ,1,.855	1

* given for "monohydroxy α-carotene-like pigment".

1) Moster, Quackenbush and Porter (1952)

2) Bickoff, Livingston, Bailey and Thompson (1954)

3) Calculated from data of Krinsky and Goldsmith (1960)

4) Calculated from data of Cholnoky, Szabolcs and Nagy (1958)

TABLE 12 Estimation of carotenoids in leaf extracts

as mg/100mg of total chlorophyll:-

1 Neo-xanthin	2 Viola-xanthin	4 Anthera-xanthin	5 Lutein	11 β-carotene	Total carotenoid*
1.84	3.18	-	4.63	4.45	13.84
2.32	5.25	-	8.40	4.51	22.39
2.61	3.42	-	6.76	4.11	16.89
2.26	4.63	-	5.89	4.76	17.54
3.14	3.45	-	6.92	4.93	18.44
3.38	5.15	1.52	7.33	6.19	24.17
3.16	3.45	0.51	6.05	5.80	18.97
2.76	4.75	0.73	6.17	5.15	19.56
2.89	4.31	1.20	6.90	4.99	20.29
as mg/100g of fresh leaf material:-					
1.35	2.33	-	3.39	3.26	10.33
1.71	3.87	-	6.20	3.39	15.11
1.86	3.87	-	4.82	2.94	12.06
1.61	3.30	-	4.20	3.40	12.51
2.42	2.66	-	5.34	3.81	14.23
2.22	3.78	1.00	4.82	4.06	15.88
2.44	2.66	0.39	4.67	4.48	14.64
2.07	3.56	0.58	4.63	3.87	14.71
1.88	2.80	0.78	4.48	3.28	16.22

* by addition

RESULTS (C)

3) IDENTIFICATION AND ESTIMATION OF QUINONES

The quinones found in this work were characterized, as described in the Experimental Section, by means of their chromatographic behaviour and their absorption spectra. Difficulty was experienced in the early part of this work because of a number of impurities which absorb ultra violet light. One of the commonest of these is benzene, which, together with other aromatic compounds, is present in many solvents. For accurate spectrophotometric work purified solvents were used as much as possible and any extracts which were taken to dryness (often resulting in concentration of the aromatic impurities) were then taken up in a little absolute alcohol and again taken to dryness to azeotrope off any lower boiling aromatics before spectrophotometry. For high degrees of purification, TLC layers were prewashed with chloroform and freshly redistilled solvents were used for developing and eluting the chromatograms.

The first quinone eluted in the gradient elution separations described earlier is phylloquinone and the absorption spectrum of column fraction II is shown in figure 55, together with that of an authentic sample of synthetic phylloquinone. It can be seen that the absorption maxima of the two spectra correspond exactly as do the relative heights of the different peaks. As well as confirming the identity of column fraction II, fig. 55 shows that the phyllo-

FIG. 55

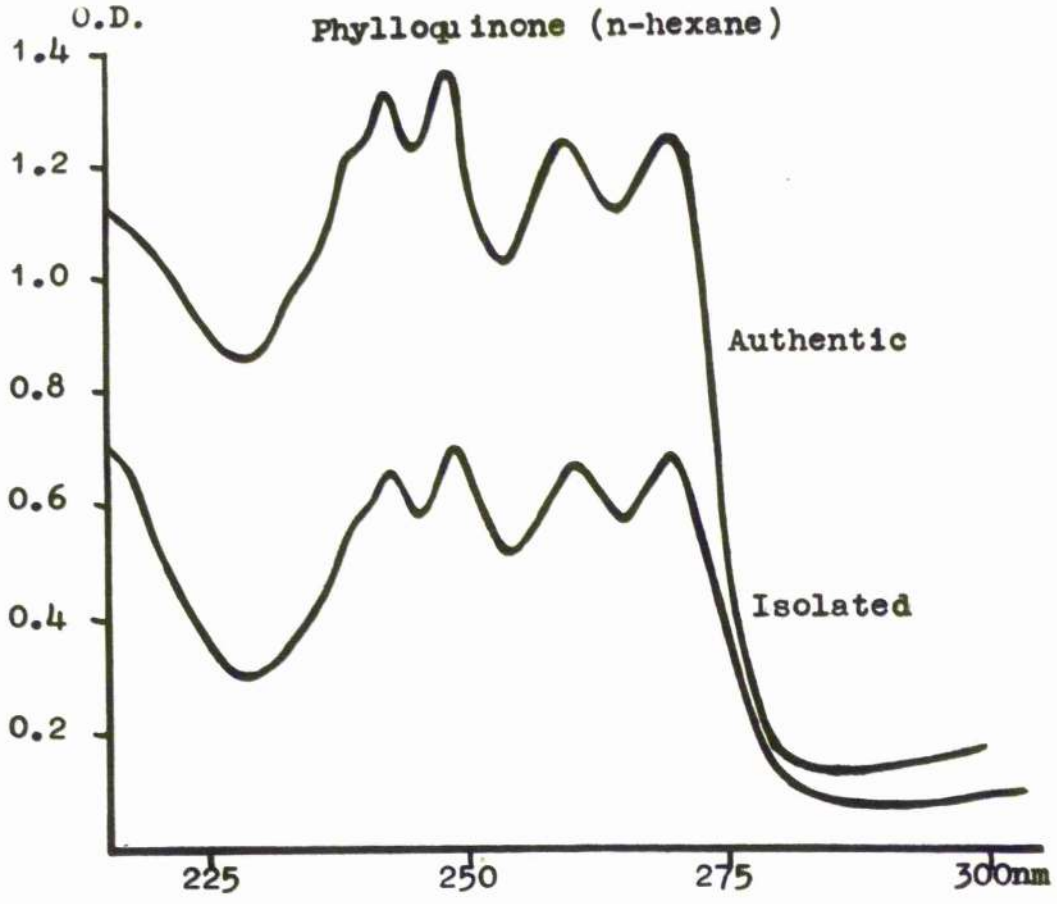
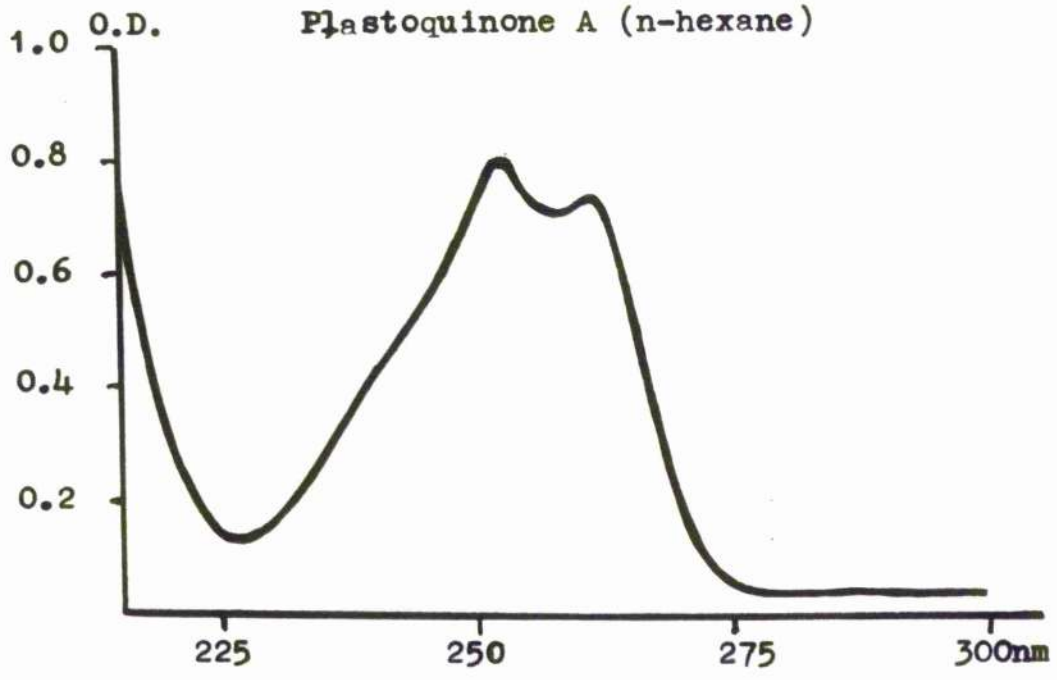

O.D.
Phylloquinone (n-hexane)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Authentic
Isolated
225
250
275
300nm

FIG. 56


O.D.
Plastoquinone A (n-hexane)
1.0
0.8
0.6
0.4
0.2
225
250
275
300nm

FIG, 57

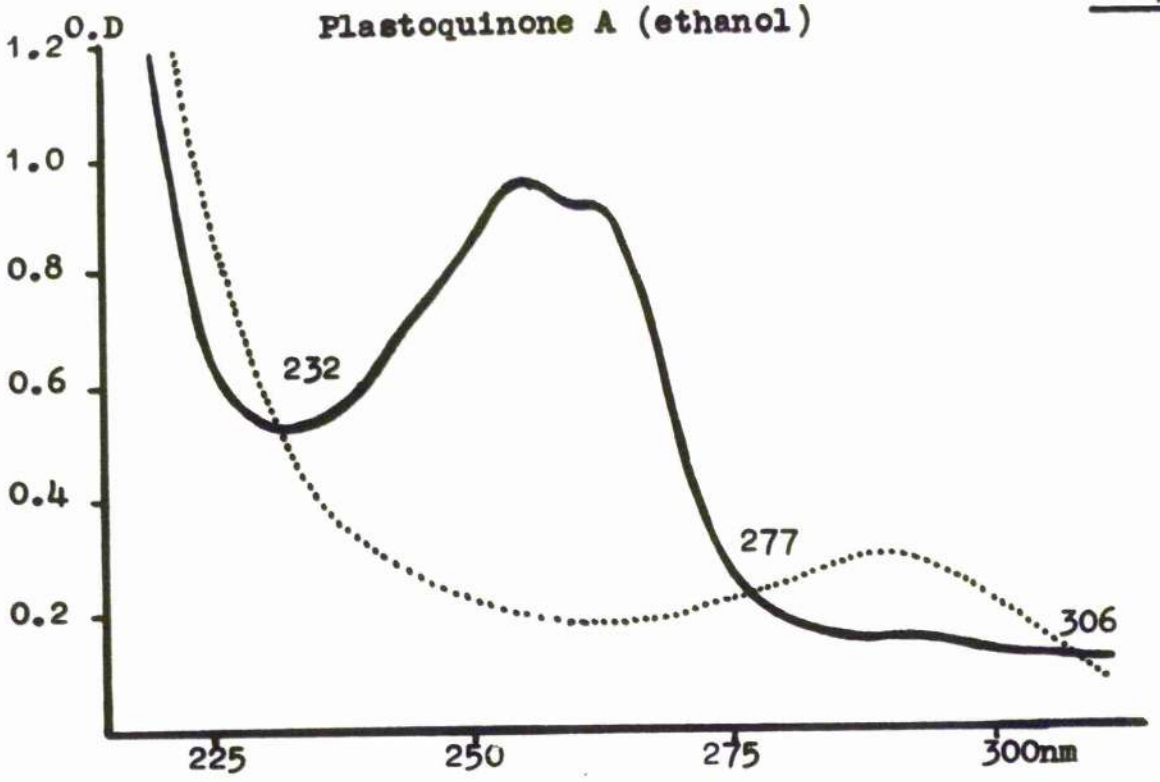

Plastoquinone A (ethanol)
O.D
1.2
1.0
0.8
0.6
0.4
0.2
232
277
306
225
250
275
300nm

FIG. 58

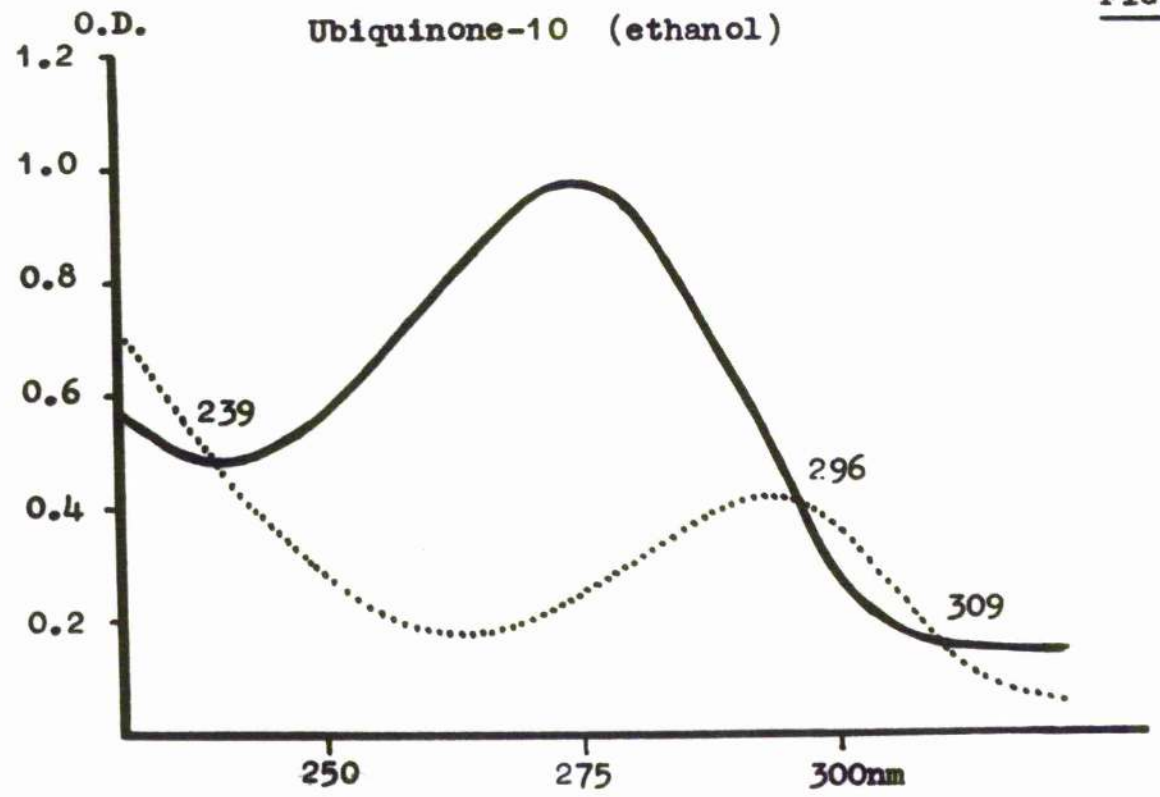

Ubiquinone-10 (ethanol)
O.D.
1.2
1.0
0.8
0.6
0.4
0.2
239
296
309
250
275
300nm

FIG. 59

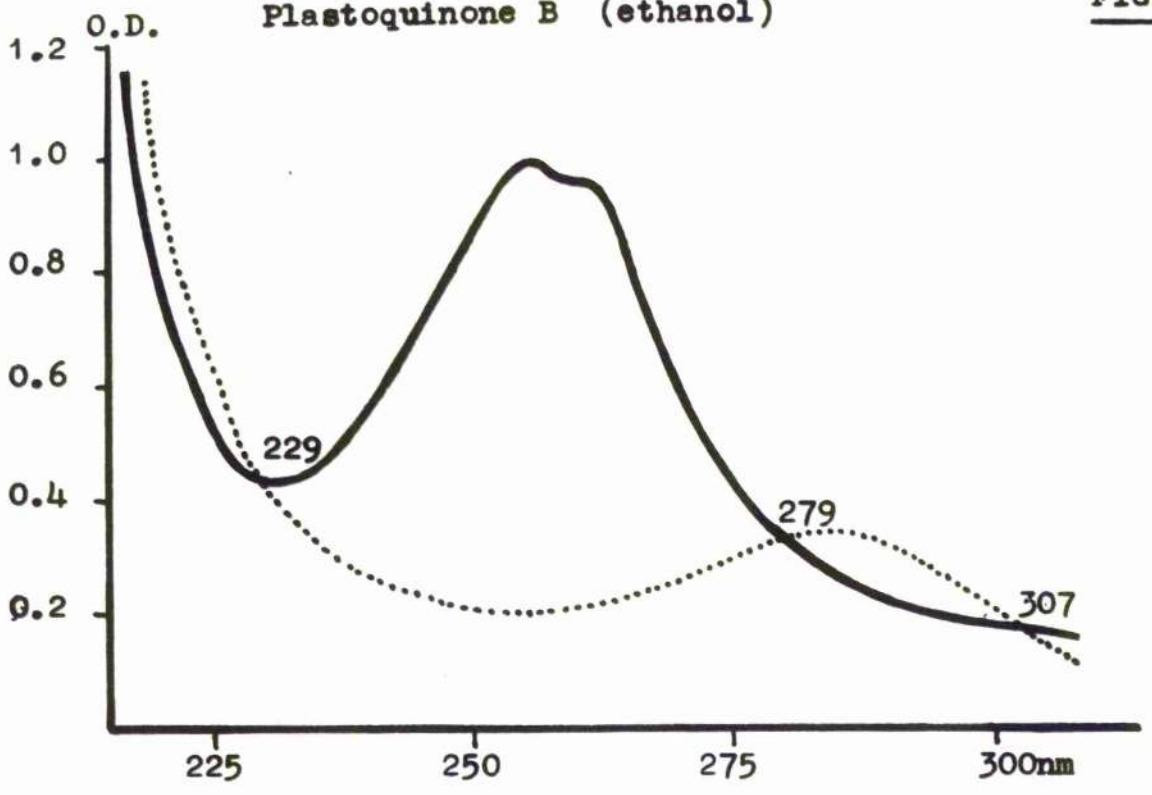
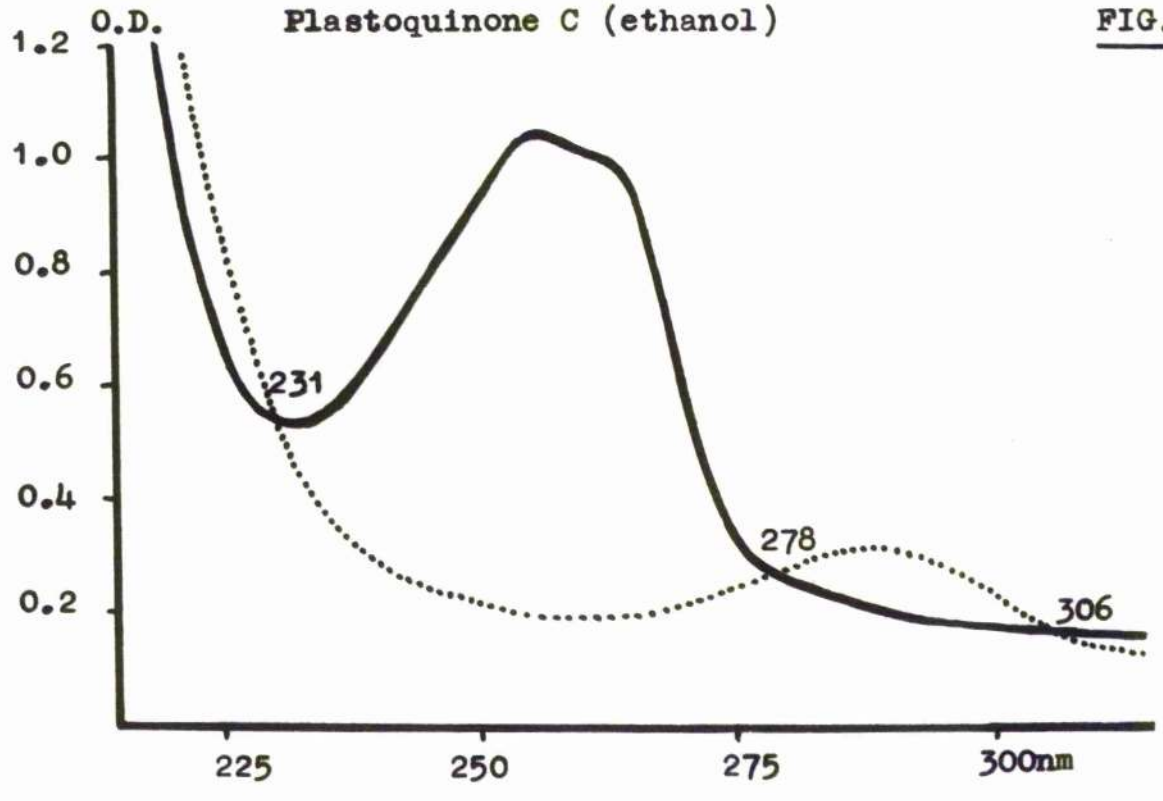

Plastoquinone B (ethanol)
O.D.
1.2
1.0
0.8
0.6
0.4
0.2
229
279
307
225
250
275
300nm

FIG. 60


Plastoquinone C (ethanol)
O.D.
1.2
1.0
0.8
0.6
0.4
0.2
231
278
306
225
250
275
300nm

quinone eluted is completely free of any ultra violet absorbing contaminants.

The major quinone of leaf extracts is plastoquinone A and the absorption spectrum of this compound, isolated by the column technique, is shown in fig. 56. The same compound is shown in fig. 57, but the solvent used in this case is ethanol and the consequent loss in resolution with a more polar solvent can be seen by comparing figs. 56 and 57. There is also a shift of the absorption maximum by 1-1.5nm to higher wavelengths in the more polar solvent. Also shown in fig. 56 is the spectrum of the reduced form of plastoquinone A, plastoquinol A, produced by adding a crystal of potassium borohydride to the plastoquinone sample.

Fig. 58 shows the oxidised and reduced spectrum of ubiquinone-10 isolated from leaf extracts. This spectrum was obtained from column fraction VI after purification by reversed-phase chromatography on polyethylene powder. The tocopherols present in the crude fraction VI led to loss of resolution in the ubiquinone spectrum, together with increased absorption in the 280-310nm region.

Figs. 59 and 60 show the comparable oxidised and reduced spectra for column fractions V and VII corresponding to plastoquinones B and C respectively. The spectrum of purified plastoquinone D was identical to that for plastoquinone D . Although the isobestic points shown in these spectra differ slightly, highly purified samples of any of the plastoquinones examined (including synthetic plastoquinone-9) all gave the

same isobestic points of 232nm and 276.5nm; a third isobestic point was also present at 306nm. Impurities in the extracts shift the isobestic points by up to 10nm in the case of the two longer wavelength points and up to 5nm in the shorter wavelength. These shifts are always away from the wavelength of maximal absorption (255nm).

Some absorption spectra were attempted directly on the column effluent without any form of concentration or purification. This ensured that the spectrum was that of the unchanged component without any degradation other than that which may have occurred on the column. A spectrum of a plastoquinone C fraction so obtained is shown in fig. 61, and since the column effluent only contains a low concentration, a 4cm pathlength cell was used (in the SP800 this required four times the normal energy to give good results). The spectrum was measured against 40% dichloroethane/n-hexane which approximated to the effluent concentration. Fig. 61 confirms the increased resolution of the spectrum in a solvent of lower polarity and also confirms that the maximum at 255nm is definitely stronger than that at 262nm.

Henninger and Crane (1964) reported that both plastoquinone C and plastoquinone D had their stronger absorption maximum at 262 rather than at 255nm, but Threlfall, Griffiths and Goodwin (1965) did not find this to be so. The spectra shown in fig. 62 may explain Henninger and Crane's results. Spectrum a) is that of a plastoquinone C + D fraction isolated

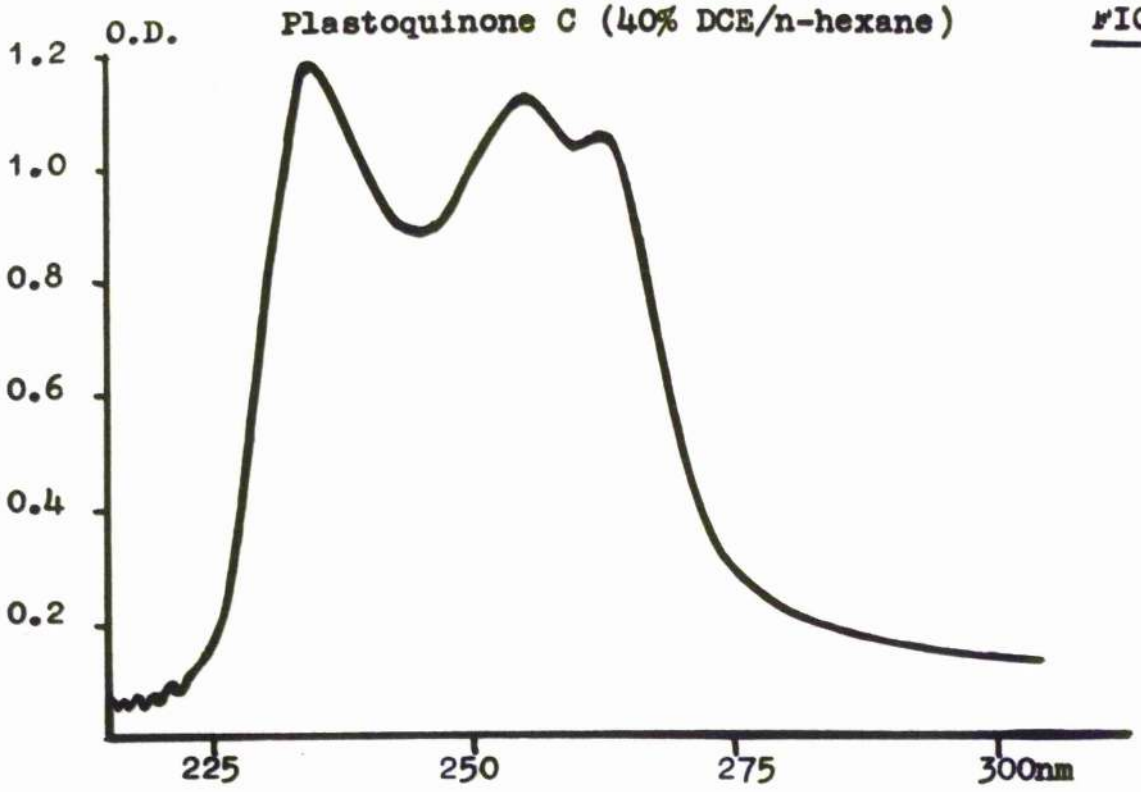

Plastoquinone C (40% DCE/n-hexane)
O.D.
1.2
1.0
0.8
0.6
0.4
0.2
225
250
275
300nm

FIG. 61

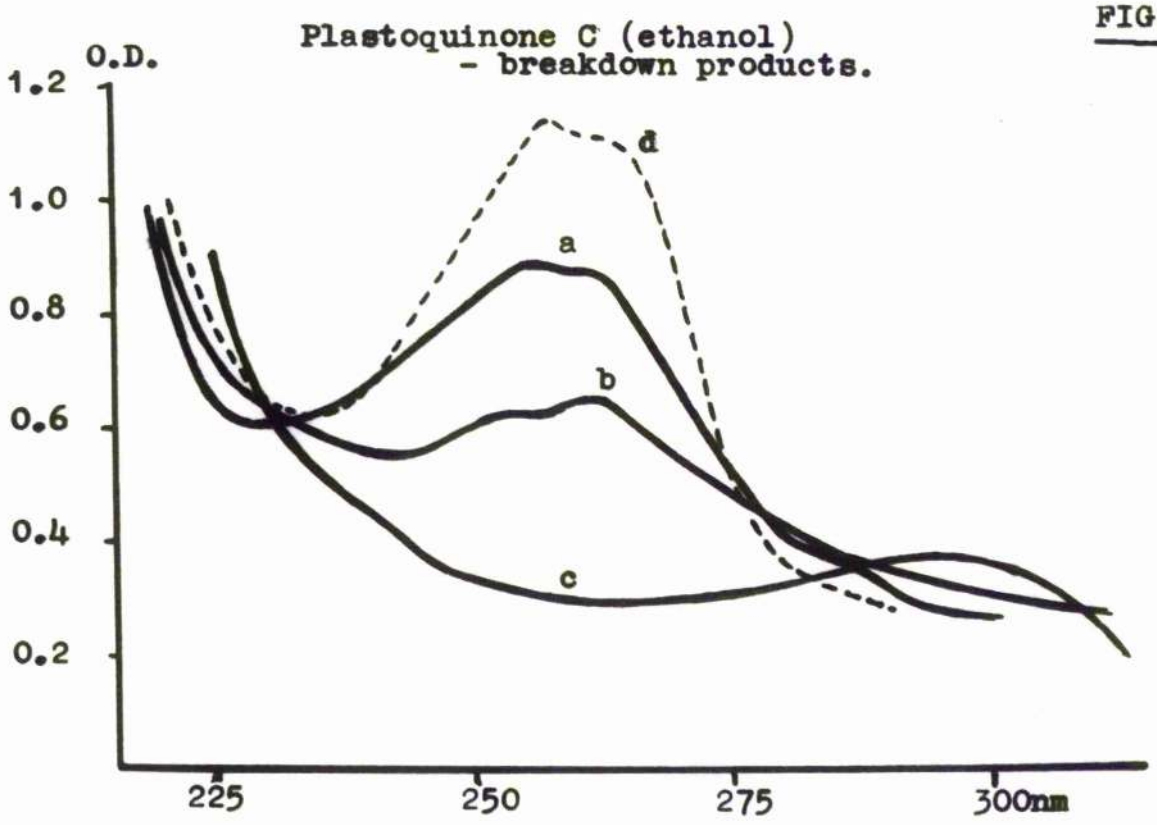

Plastoquinone C (ethanol)
- breakdown products.
O.D.
1.2
1.0
0.8
0.6
0.4
0.2
a
b
c
d
225
250
275
300nm

FIG. 62

by TLC, and spectrum b) is the same sample after standing in the open for 15 minutes. Curve c) is the spectrum obtained by reduction of this sample. As can be seen, the optical density of the sample has dropped on standing, and the 255nm maximum has become weaker than that at 262nm. In this form (curve b)) the spectrum resembles that reported by Henninger and Crane (1964) for the plastoquinones C and D and is presumably due to their partial conversion into a product with an absorption maximum around 262nm. This compound may be similar to R-263 reported by Henninger, Barr and Crane (1966) as being a breakdown product of plastoquinone B. Considering the known structure of plastoquinone B as an ester of plastoquinone C and/or D, it is possible that R-263 may also be a breakdown product of plastoquinone C and D. The dotted line d) in fig. 62 is suggested as approximating to the spectrum of the original undegraded plastoquinone (by comparison with fig. 60).

Figs. 63 and 64 show the absorption spectrum of α-tocopherolquinone, isolated from leaves, in hexane and ethanol respectively. Fig. 64 also shows the spectrum of the quinol formed on reduction of α-tocopherolquinone with a crystal of potassium borohydride and isobestic points are seen at 230nm and 283nm as compared with 231 and 281nm (Lichtenthaler and Calvin, 1964), and 232 and 283nm (Carr and Hallaway, 1965). The third isobestic point is around 306nm, but the oxidised and reduced spectra come together at this point, so it is

FIG. 63

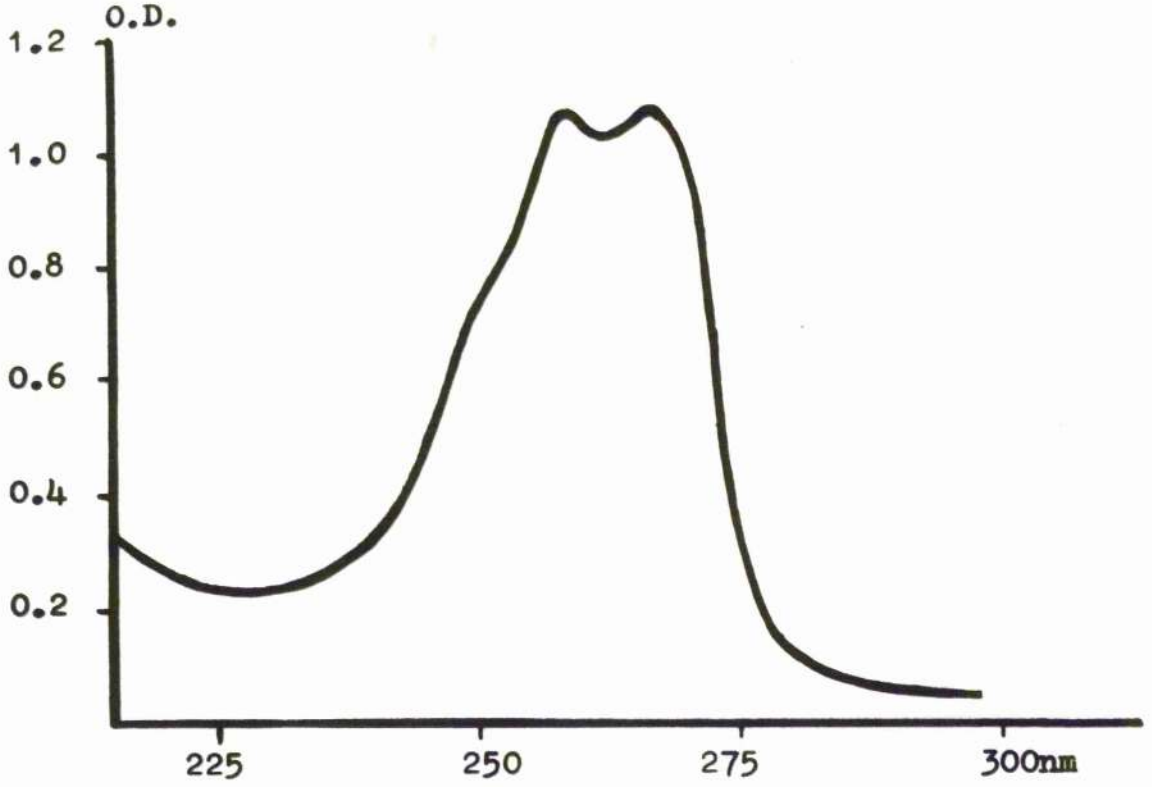
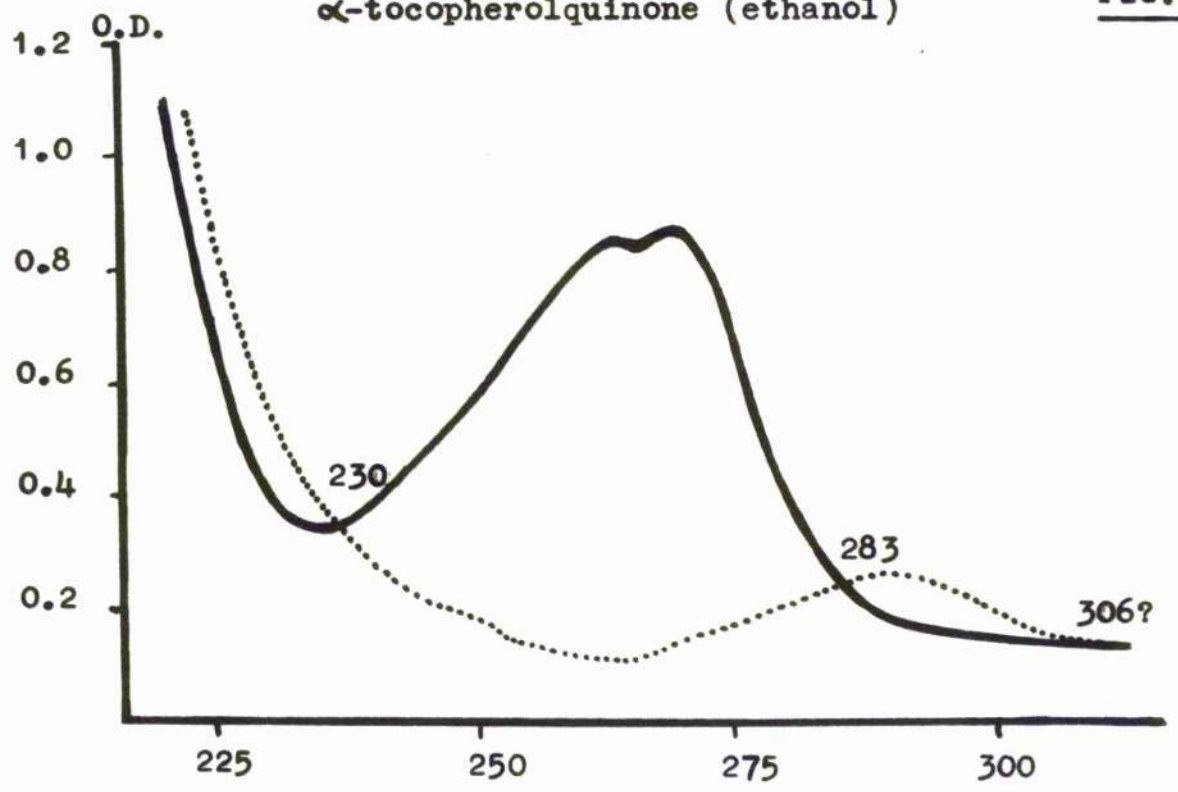

α-tocopherolquinone (n-hexane)
O.D.
1.2
1.0
0.8
0.6
0.4
0.2
225
250
275
300nm

FIG. 64


α-tocopherolquinone (ethanol)
O.D.
1.2
1.0
0.8
0.6
0.4
0.2
230
283
306?
225
250
275
300

difficult to tell. It can be seen that the absorption maxima in the non-polar solvent n-hexane (fig. 63) are sharper and at a lower wavelength than in ethanol, each peak being displaced by 2nm. This effect is similarly observed in the plastoquinons and ubiquinones.

The concentrations of all the quinones were estimated spectrophotometrically, but were found to vary during the growing season. The concentrations found are reported in Results Section E and the average values are discussed in the Discussion at the end.

RESULTS (C)

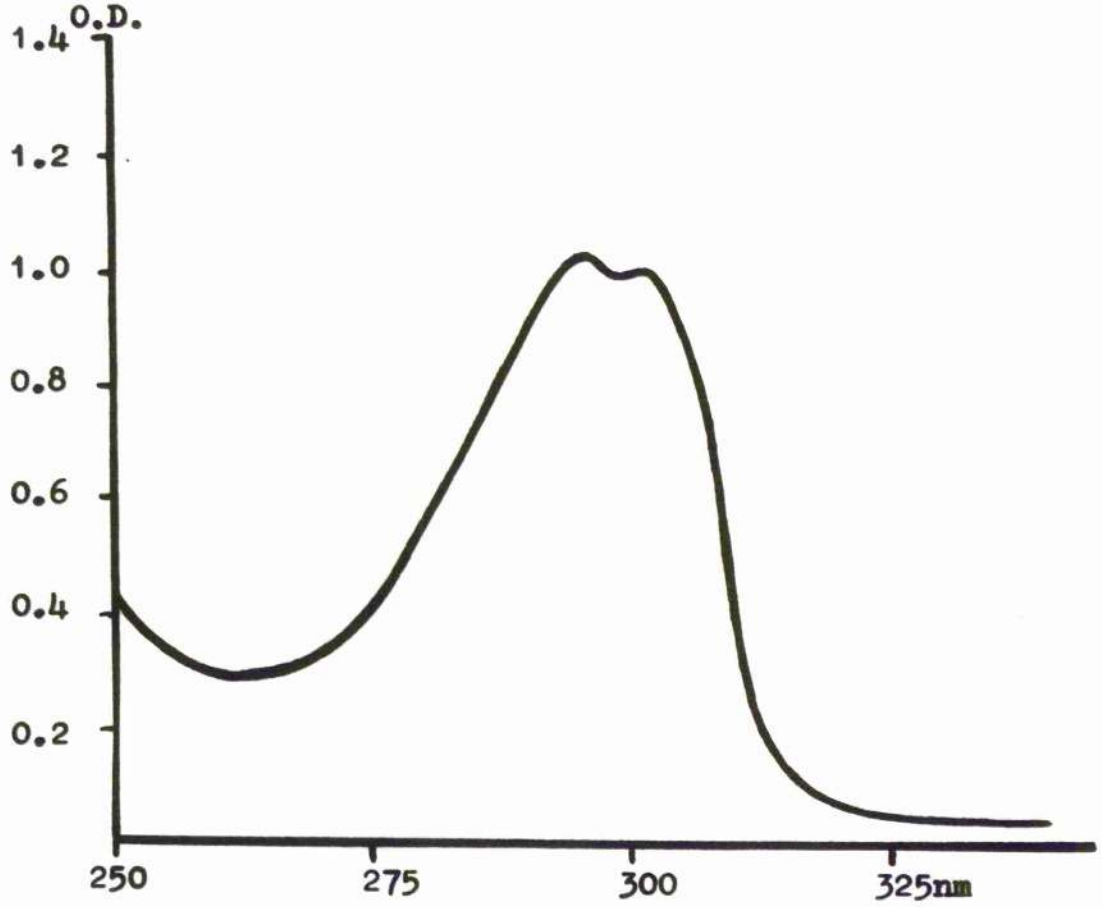
4) IDENTIFICATION OF PLASTOCHROMANOL

Plastochromanol was the only chromanol of which the spectrum was studied in this work and its ultraviolet absorption spectrum is shown in fig. 65. As can be seen, this compound exhibits a maximum at 294.5nm, and a second, slightly lower one at 300.5nm, as compared with 294 and 300.5nm as reported by Whittle, Dunphy and Pennock (1965).

The concentration of plastochromanol found in the leaves of spinach beet was about 3.6mg/100g fresh weight, as compared with the value of 7.3mg/100g reported by Dunphy, Whittle and Pennock (1966) for this plant.

FIG. 65

Plastochromanol (n-hexane)



RESULTS (D)

ETIOLATION EXPERIMENTS

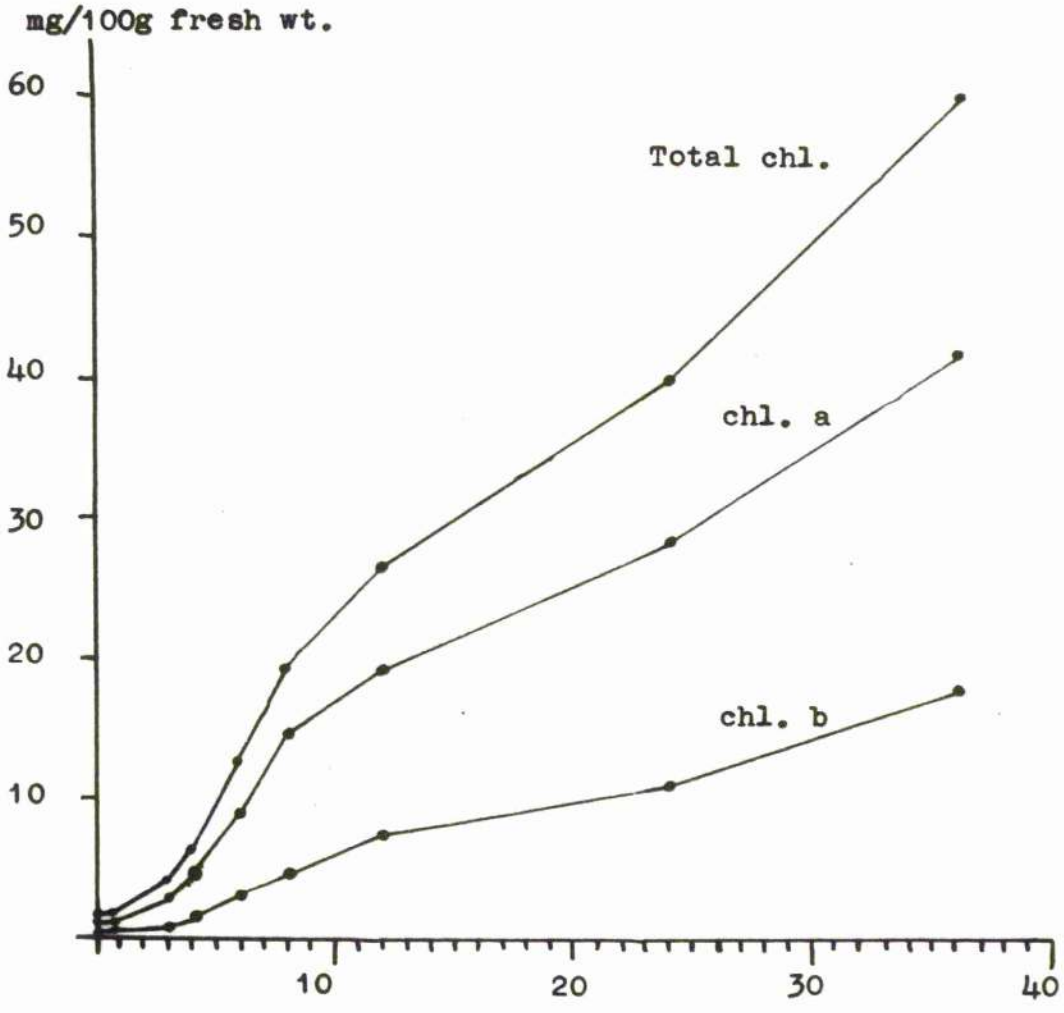
1) DEVELOPMENT OF THE CHLOROPHYLLS

Traces of both chlorophyll a and chlorophyll b were found in the etiolated seedlings examined; the concentrations found were 0.75 and 0.36mg/100g fresh weight respectively. The chlorophyll a present could have arisen by a rapid conversion from protochlorophyll but the presence of chlorophyll b suggests that the seedlings may have received a trace of light at some time during their growth.

Fig. 66 shows the development of the chlorophylls on subsequent illumination, and the early results are the mean of two experiments which duplicated each other almost exactly. It can be seen that the synthesis of chlorophyll a started after about 30 minutes, whilst chlorophyll b synthesis did not start until after about 3 hours illumination. There was a rapid synthesis of chlorophyll a in the first 8 or 9 hours and the rapid synthesis of chlorophyll b continued for 3 or 4 hours longer, possibly reflecting the initial delay in the commencement of chlorophyll b synthesis. After this time there was a somewhat similar increase in both the chlorophylls up to the end of the experimental time of 36 hours of illumination. The final chlorophyll content was 59.6mg/100g leaf which is between one third and one half of the value expected for mature leaves. The ratio of chlorophyll a to chlorophyll b is 2.4:1, which may reflect what is possibly a

Fig. 66

Development of chlorophylls in 5 day old etiolated seedlings on illumination



relatively low light intensity.

RESULTS (D)

2) DEVELOPMENT OF THE CAROTENOIDS

The etiolated cotyledon leaves examined were found to contain 68.1% of their carotenoids as lutein and only 7.6% as carotene. Since the carotene fractions absorption spectrum was not identical to that of β-carotene, as regards the relative optical density of its maxima, it is possible that some other non-polar carotenoids, such as zeacarotenes may be present; alternatively, a high proportion of α-carotene may be present. Table 13 shows that violaxanthin accounted for 9.9% of the total carotenoids, but about one tenth of this was present as its furanoid derivative auroxanthin, and similarly, about one quarter of the neoxanthin was actually found as mutatoxanthin. Since furanoids are easily formed by acidic conditions, the furanoids found are included with the parent epoxy carotenoid in the results shown. A small amount of antheraxanthin and zeaxanthin were also found to be present.

As can be seen from fig. 67, there is very little carotenoid synthesis in the first four or five hours, apart from the apparent increase in zeaxanthin; this could have been due to contamination by some other pigment, but this is unlikely; the zeaxanthin content was zero after a further four hours. Neoxanthin is also synthesized in the first few hours of illumination and the amount formed is very similar to the decrease in β-carotene in this time.

Fig. 67

Development of carotenoids

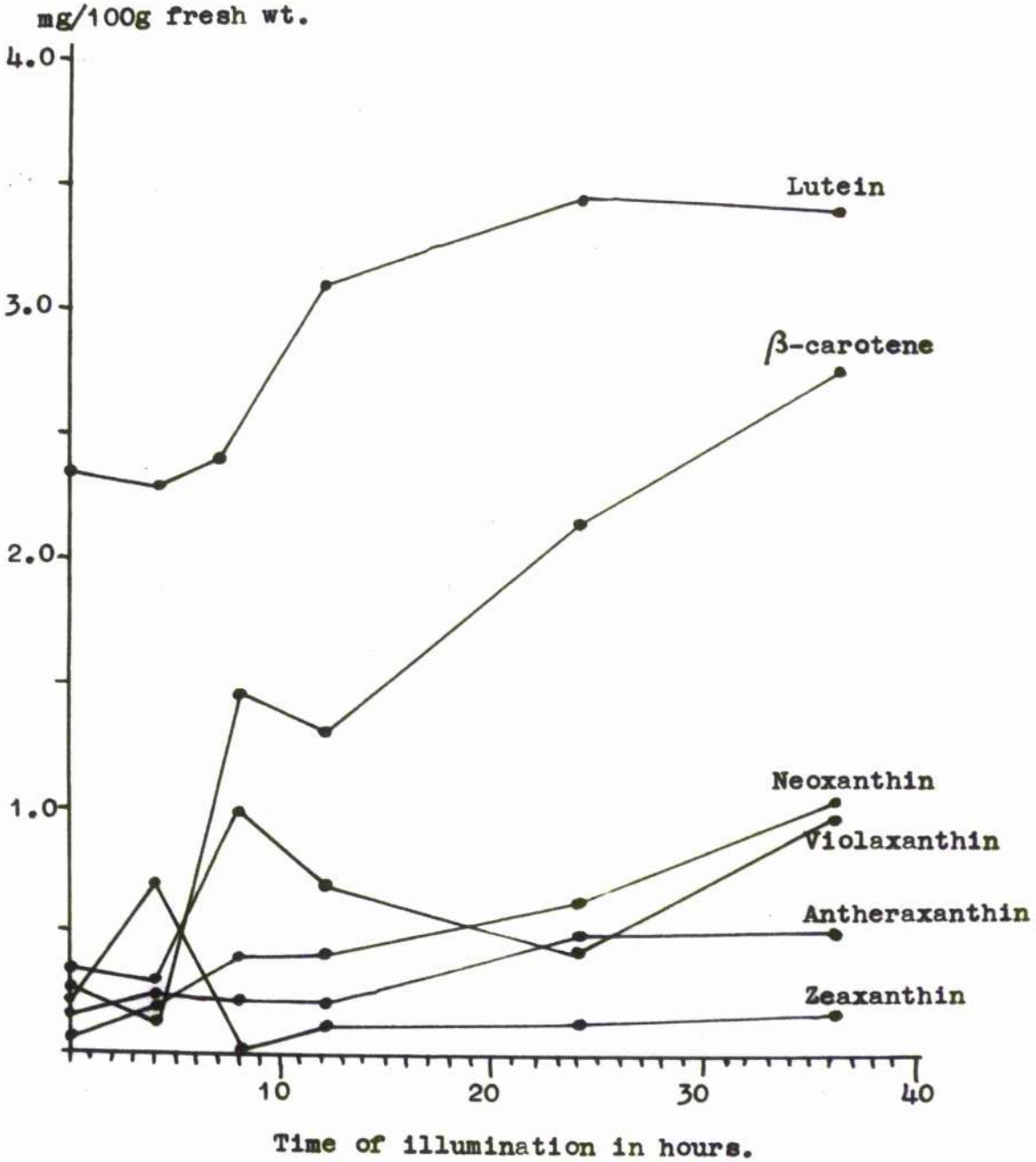


Fig. 68

Development of total chlorophyll and carotenoids

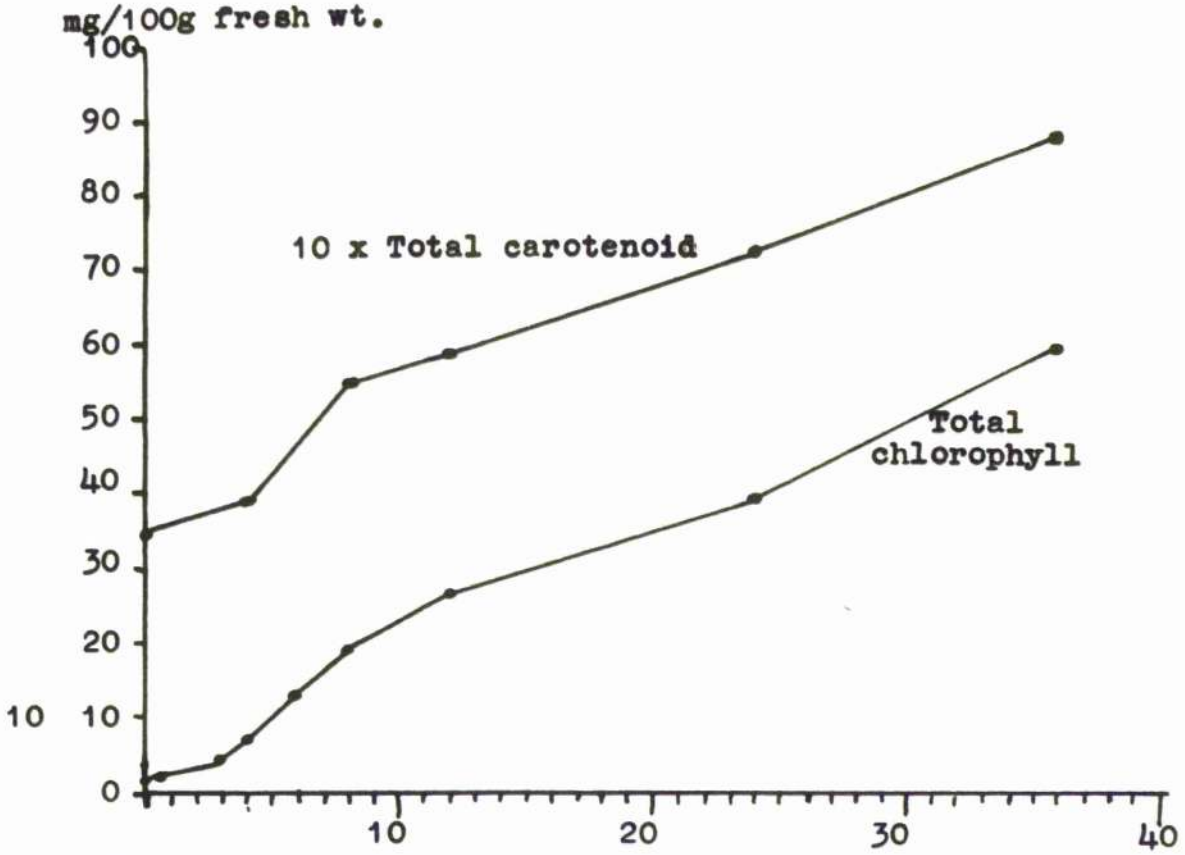
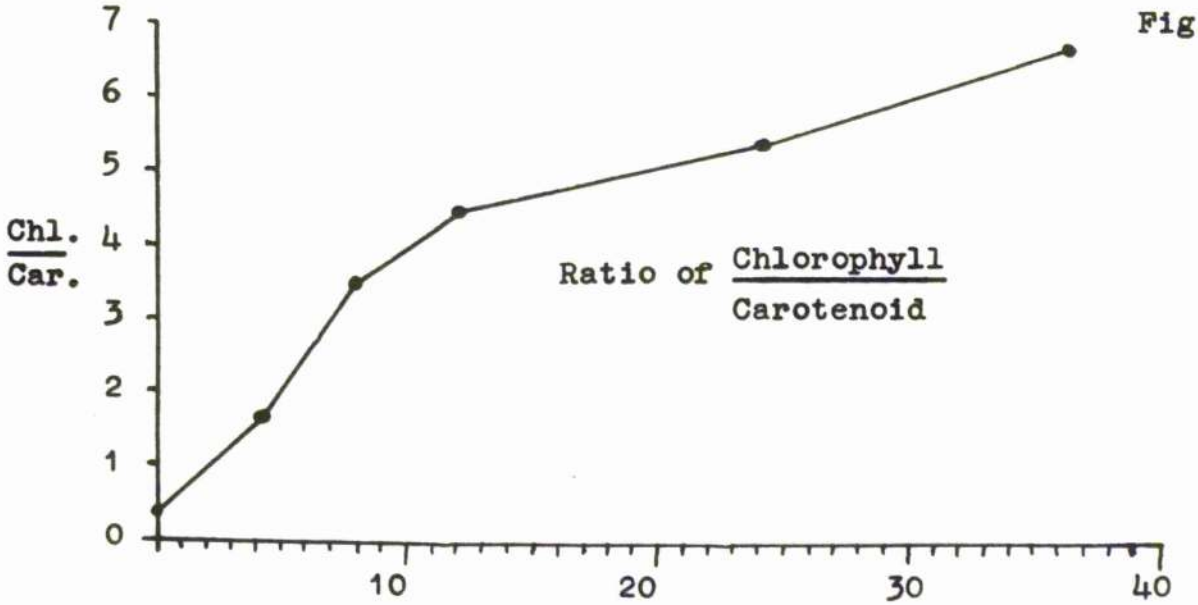


Fig. 69



Time of illumination in hours

After the first four or five hours a rapid synthesis of violaxanthin and β-carotene ensues and the increase in neoxanthin continues. After a further four hour period (8-9 hours illumination) the weight of β-carotene present in the leaves appears to drop temporarily and this is accompanied by a drop in violaxanthin, which continues for another sixteen hours. At the same time as the β-carotene content drops, the lutein content starts to increase, possibly suggesting that the drop in carotene is actually a decrease in the α-isomer. With the resumed synthesis of β-carotene, the two major leaf pigments, β-carotene and lutein are synthesized at a somewhat similarrate up to about 24 hours of illumination when lutein synthesis seems to stop.

During the first 24 hours there is a steady increase in neoxanthin, both with regard to its total weight and as regards its concentration as a percentage of the total carotenoid present. Antheraxanthin also shows an increase with regard to its total weight, but its concentration with respect to the total carotenoid content has increased only slightly. The zeaxanthin content remains fairly constant on both the above counts after its initial rise and decrease.

The synthesis of β-carotene and neoxanthin continues at a similar rate up to the end of the 36 hour period of illumination and in the last twelve hours violaxanthin is synthesized at a similar rate. At the end of this time the concentration of the individual carotenoids, as a percentage of the total, are approaching the expected values for

mature leaves (see table 13), although the violaxanthin is still somewhat low.

α- and β-cryptoxanthin have not been included either in fig. 67 or in this summary of results since both are minor pigments which, under these experimental conditions, remain fairly constant with respect to their percentage of the total carotenoids.

TABLE 13 Development of individual carotenoids as % total

	0 hrs	4 hrs	8 hrs	12 hrs	24 hrs	36 hrs
β-carotene	7.6	3.1	26.3	22.3	29.2	31.0
α-cryptoxanthin	1.4	1.3	0.9	0.9	0.9	0.8
β-cryptoxanthin	-	-	0.2	0.3	0.3	0.3
Lutein	68.1	59.5	43.4	52.7	46.8	38.2
Antheraxanthin	4.6	6.2	3.8	3.4	6.6	5.6
Zeaxanthin	6.4	17.5	-	1.9	1.7	1.8
Violaxanthin	9.9	7.2	18.1	11.7	6.0	10.9
Neoxanthin	2.0	5.2	7.3	6.8	8.5	11.4

Figs. 68 and 69 show the development of the total carotenoid with respect to that of chlorophyll. In fig. 68 it should be noted that the carotenoid content is graphed as ten times the total carotenoid, thus the parallelism of the two pigment curves means that chlorophyll is being synthesized about ten times as fast as the carotenoids. The ratio of total chlorophyll to carotenoid is shown in

fig. 69 and the 36 hour value of 6.7 lies in the region expected for normal mature leaves (about 6:1 to 9:1).

N.B. Some of the antheraxanthin fractions examined in this work showed the presence of a mono-epoxide with absorption maxima about 5nm shorter than those of antheraxanthin. This compound may have been lutein epoxide.

RESULTS (D)

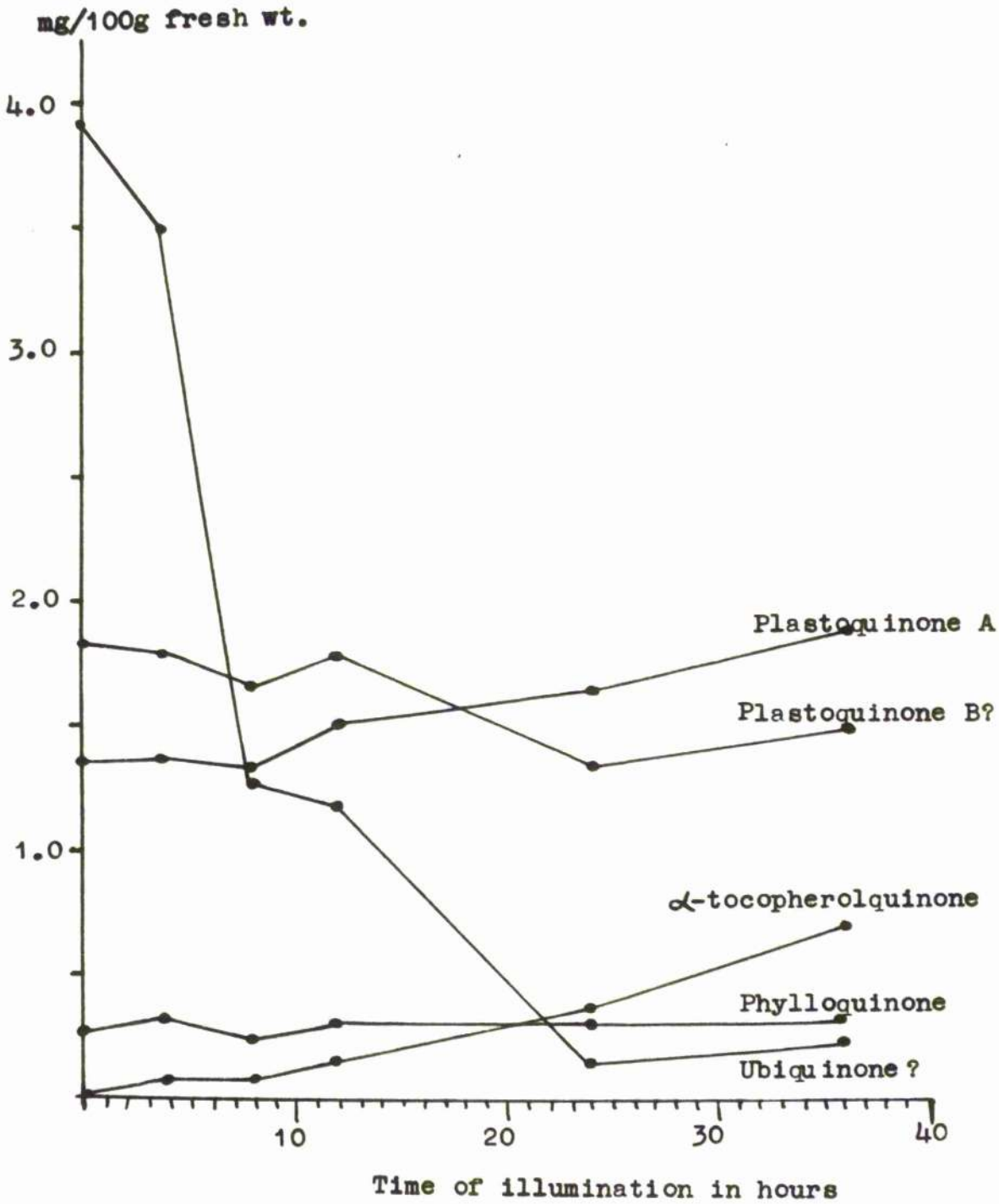
3) DEVELOPMENT OF THE QUINONES

Fig. 70 shows the development of the isoprenoid quinones on illumination of etiolated radish seedlings. The curves for ubiquinone and plastoquinone B are queried because the results for ubiquinone seem somewhat unexpected and the identity of these column fractions was not checked by further analysis. Hence it is possible that some unknown compound has so distorted the column separation in the ubiquinone region that ubiquinone is running in the expected position of plastoquinone B. In this case the "plastoquinone B?" curve corresponds to ubiquinone (but the concentration is approximately twice that shown due to their differing extinction coefficients) and the "ubiquinone?" curve would correspond to some unknown ultraviolet absorbing component. By comparison with other work (Gaunt and Stowe, 1967; Griffiths, Threlfall and Goodwin, 1967) the ubiquinone content would be expected to remain constant, although no work has been reported on the cotyledon leaves of dicotyledonous plants. If the "plastoquinone B?" curves does, in actual fact, correspond to ubiquinone, then it is uncertain whether or not this fraction also contains plastoquinone B.

In the cotyledon leaves examined, under the light intensity used, there is little or no synthesis of phylloquinone, again this result does not agree with other work on monocotyledons (Griffiths, Threlfall and Goodwin, 1967)

Fig. 70

Development of Quinones



or on apical buds of pea seedlings (Gaunt and Stowe, 1967). Similarly the increase in plastoquinone A on illumination is not as great as that reported by these other workers. These facts, taken together with the queries concerning ubiquinone, show that comparisons with other systems may not be valid and thus the curve "ubiquinone?" in fig. 70 may indeed correspond to ubiquinone.

In agreement with the work of Griffiths, Threlfall and Goodwin (1967) on etiolated maize and barley shoots, no α-tocopherolquinone could be detected in the etiolated tissue examined. As found by these workers however, α-tocopherolquinone was synthesized on illumination and this can be seen in fig. 70.

The final concentrations of the quinones, with respect to chlorophyll, after 36 hours of illumination, were: phylloquinone, 0.575mg/100mg; plastoquinone A, 3.17mg/100mg; α-tocopherolquinone, 1.18mg/100mg.

RESULTS (E)

SEASONAL VARIATION OF THE QUINONES

The concentration of the various quinones in spinach beet leaves was measured throughout the season by means of the gradient elution technique described in Experimental Section B 1) and the results obtained are plotted in figs. 71 and 72. Although the quinone estimations were performed fairly often, usually three times or more in any one week, for simplicity the average values for half monthly periods are shown in the figures.

As can be seen, all the plastoquinones increase during the growing season, but although plastoquinones A and C both show an increase with respect to chlorophyll, the plastoquinone B content remains approximately constant when considered with respect to the chlorophyll concentration (average value of 0.88mg/100mg of chlorophyll). As expected, the ubiquinone content correlates more closely with the fresh weight of the leaf than with the chlorophyll content. This result is in keeping with the different sites of ubiquinone and chlorophyll in the mitochondria and chloroplasts respectively.

Phylloquinone shows a slight rise during the summer months, but this increase cannot be seen in fig. 72, and so the phylloquinone content correlates very closely to that of chlorophyll. It should be noted that the leaves examined in this work show a fairly low phylloquinone content,

Fig. 71

Variation of isoprenoid quinones throughout the growing season

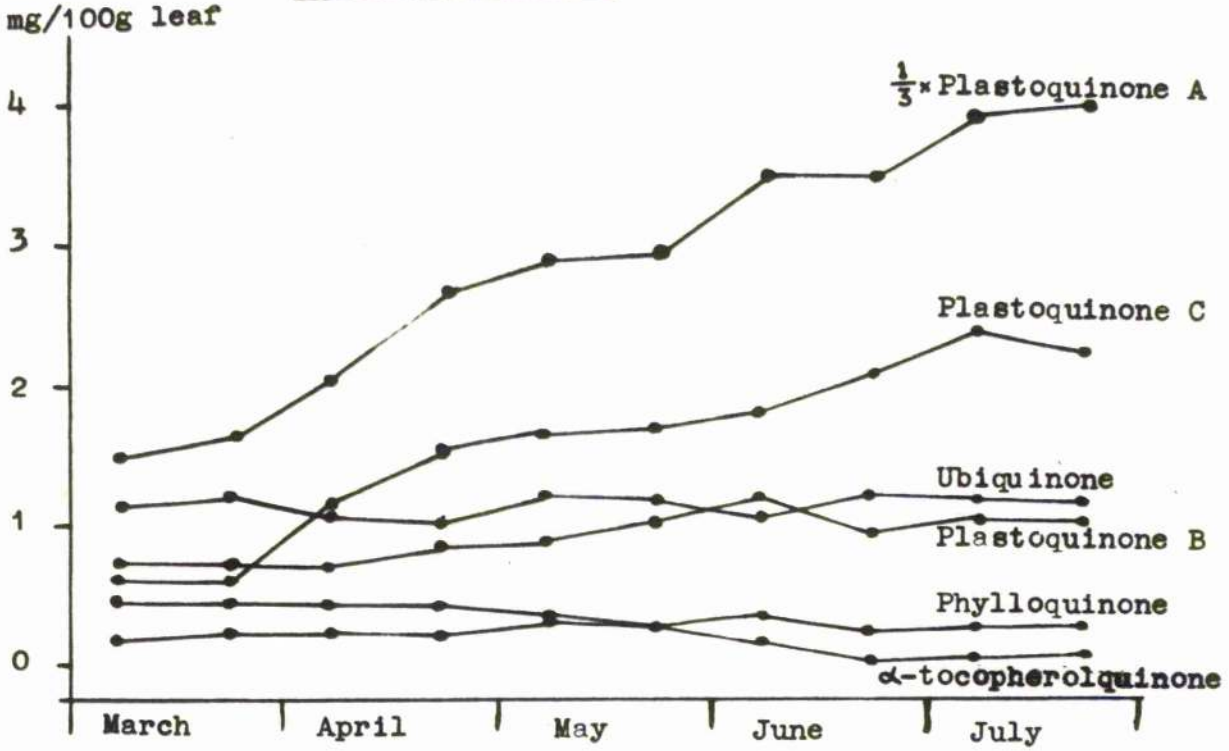
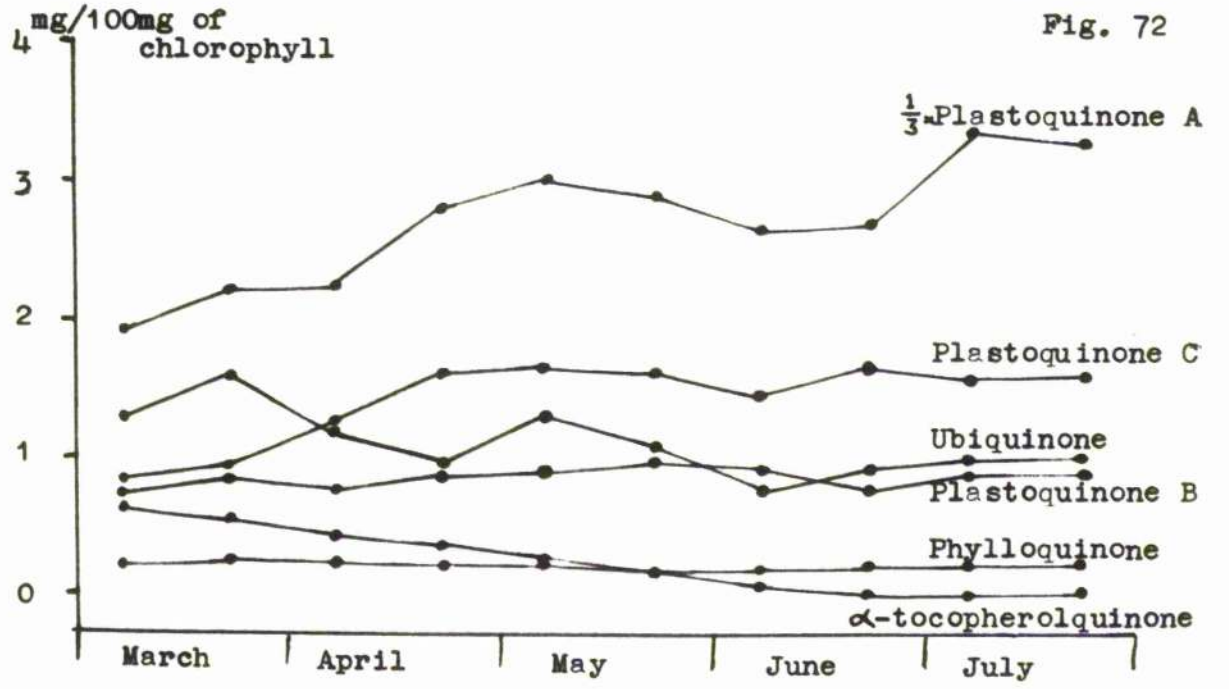


Fig. 72



only about 0.25mg/100mg of chlorophyll.

In contrast to the other quinones above, α-tocopherolquinone shows no correlation either with fresh weight or chlorophyll content, in fact, the α-tocopherolquinone concentration decreases with the increase in chlorophyll during the summer months.

Plastoquinone D was normally found in fresh leaf extracts at between one half and two thirds of the concentration of the plastoquinone C present, and both these compounds exhibited a similar increase in concentration during the summer months. Bucke and Hallaway (1966) have noted an inverse relationship between plastoquinone C and α-tocopherolquinone, and this effect can be seen in figs. 71 and 72. During the winter months the ratio of plastoquinone C to α-tocopherolquinone is between 1:1 and 2:1, whilst in the summer it may rise to 20:1 and α-tocopherolquinone may even be undetectable. Plastoquinone C and D were both detectable during the winter, in contrast to the findings of Bucke and Hallaway (1966) that plastoquinone C could hardly be detected in winter grown leaves. As suggested in the Discussion following, this may have been due to the low light intensity available to their plants which were grown indoors during the winter.

The results of this study on the seasonal variation of the plant quinones tend to modify the conclusions of Crane, Henninger, Wood and Barr (1966) that "all the quinones tend to shift together so that the relative amounts remain

the same in relation to the other quinones". As shown in figs. 71 and 72, whilst plastoquinones A and C tend to shift together, plastoquinone B and phylloquinone remain fairly constant and α-tocopherolquinone exhibits an inverse relationship.

The minor column fractions IIA and VIIIA, corresponding to demethylated phylloquinone and $\beta+\gamma$ tocopherolquinone respectively, were not fully studied. Fraction IIA was only occassionally observed and there was no apparent seasonal or light intensity effect on its appearance. Fraction VIIIA, $\beta+\gamma$ tocopherolquinone was only present at a very low concentration, usually between one tenth and one twentieth of the concentration of α-tocopherolquinone, and appeared to exhibit similar seasonal changes.

DISCUSSION

DISCUSSION

The methods of extraction of the lipids from plant leaf material utilised in this work (particularly method b) in the Experimental Section) appear to give an efficient extraction of the pigments and the relatively non polar lipids studied. According to Thirkell (1961) the "Impulse Rendering Process" also gives an efficient extraction of leaf lipids and this latter process is more feasible for use on a commercial scale.. Unfortunately, the samples of commercially prepared leaf extract studied in this work (prepared by British Glues and Chemicals Ltd., utilising the Impulse Rendering Process) showed a large number of chlorophyll breakdown products which, to a great extent, obscured the xanthophylls in all the methods of separation attempted. The β-carotene content of such commercial preparations was also relatively low in comparison with leaf extracts prepared during this work. Both the presence of chlorophyll degradation products and the apparent loss of β-carotene no doubt reflect the adverse conditions operative in the drying procedure of such commercial preparations: azeotropic drying with toluene at temperature up to 106°C (Thirkell, 1961). If this drying procedure could be conducted without degradation of the leaf pigments, then the analysis of such extracts would no doubt yield results substantially the same as those of the present work

with fresh leaf extracts prepared in the laboratory (by the methods described in the Experimental Section).

The separation of the lipids present in leaf extracts has been attempted by a number of chromatographic techniques as described in the Experimental Section, and for the best results some initial purification step was found to be advisable. Saponification has sometimes been used in the examination of the carotenoids present in leaves but this procedure has been avoided in any examination of the quinones because of its possible degradative action. The preliminary purification normally utilised, consisted of removing the polar lipids which were not examined in this study. These compounds were removed either by precipitation of the waxes and phospholipids from their acetone solution or else by washing a hexane solution of the leaf lipids with 80% and 90% methanol, to remove the majority of the polar lipids present. In this latter method it was found advisable to equilibrate the aqueous methanol with hexane before use. The washing procedure still removed a small amount of the chlorophyll present however, more of the chlorophyll b being removed than of the less polar chlorophyll a. Consequently, any chlorophyll estimations were performed before any purification of the leaf extract was attempted.

The major chromatographic technique used throughout this work was thinlayer chromatography (TLC) and this gave excellent separations of many of the lipids present in leaf

extracts. In addition to the compounds mainly studied in this work, the quinones and carotenoids, TLC was used to show the presence of a number of other components including the hydrocarbons squalene and phytoene; another, presumably saturated, hydrocarbon was also found running ahead of squalene. Sterol esters and waxes were also found by TLC, together with a somewhat unstable wax-like component running ahead of the sterol ester fraction. The sterol ester fraction was obviously heterogeneous from the number of partially resolved spots that could be seen on TLC plates.

The chromanols present in leaves have also been examined by TLC and the presence of α -tocopherol has been confirmed together with a mixture of β- and γ -tocopherols. Small amounts of δ -tocopherol have also been shown to be present. In the course of this examination of the tocopherols an additional chromanol has been isolated which gives a blue-grey colouration with the diazo reagent (see Experimental Section B,2)d)(xi)). This compound has been shown to have spectral and chromatographic properties of plastochromanol-8, the cyclic derivative of plastoquinone A reported by Whittle, Dunphy and Pennock (1965) and Dunphy, Whittle and Pennock(1966). The concentration of plastochromanol found in the leaves of spinach beet (about 0.36mg/100g leaf) is about half of that reported by Dunphy, Whittle and Pennock (1966) for the same material. Two other compounds have also been observed which, from their reaction with the

diazo reagent, appear to be phenolic in nature and these have been tentatively identified as quinone precursors similar to those described by Olson (1966) and Rudney and Raman (1966).

TLC on silica gel has been used to separate the carotenoids of plant leaves and using a solvent of acetone/hexane (40/60) a routine assay method for the carotenoids has been developed. Several of the values obtained are reported in table 12 of the Results Section and the average of these is presented in table 14 below, together with the values reported by other workers for comparison.

Table 14 Quantitative analysis of the major carotenoids present in leaf extracts.

Compound	mg/100mg of chlorophyll		mg/100g fresh leaf		Carotenoids as % of total carotenoid		
		(1)		(2)		(1)	(2)
β-Carotene	5.03	3.7	3.6	6.06	26.8	28.5	28.9
Lutein	6.8	5.9	4.9	8.8	37.0	45.5	41.8
Violaxanthin	4.1	1.8	3.0	4.0	22.0	13.7	19.1
Neoxanthin	2.7	1.6	2.0	2.1	14.4	12.3	10.2
Total carotenoids*	18.5	12.9	13.6	20.9	100	100	100

(1) Lichtenthaler and Calvin (1964)

(2) Yamamoto, Nakayama and Chichester (1962)

* By addition.

As can be seen, the results obtained for the various carotenoids are in better agreement with those of Yamamoto,

Nakayama and Chichester (1962) than with those of Lichtenthaler and Calvin (1964), at least with regard to their percentage of the total carotenoids. The concentration of the lutein fraction found in this work appears to be slightly low, whilst that of the neoxanthin fraction is slightly high. The same workers have however reported neoxanthin contents of 12.6% of the total carotenoids in spinach chloroplasts and up to 17.7% in the alga Chlorella (Yamamoto, Chichester and Nakayama, 1962a, 1962b). The minor carotenoids are not included in table 14, but they appear to consist of α-cryptoxanthin (0.8%), β-cryptoxanthin (0.3%) and antheraxanthin (3-4%). The antheraxanthin fraction may sometimes include zeaxanthin, but these two compounds are normally separated on TLC in the system used. Consequently, when only one carotenoid band was observed in this region it seems likely that it was antheraxanthin. When zeaxanthin was found it usually represented less than 1% of the total carotenoids. Evidence from the etiolation experiments (Results Section D 2)) suggests that the antheraxanthin fraction may have been contaminated with xanthophyll epoxide, although whether this is so in mature leaves is uncertain.

The technique described has been shown to have a reproducibilty of better than $\pm$ 5% and so is suitable for studying the changes in leaf carotenoids under different physiological conditions. The assay method has been used in this work to study the development of the individual

carotenoids on illumination of etiolated leaves. The results of this work are shown in fig. 67 and table 13 in the Results Section. As can be seen, very little furanoxy carotenoid was found in the dark-grown radish cotyledons used, in contrast to the report of Goodwin and Phagpolngarm (1960) that the carotenoids of etiolated bean cotyledons contain high concentrations of furanoxides. Since epoxy carotenoids are converted to their corresponding furanoxy derivatives by traces of acid it is possible that bean cotyledons may contain a higher acid content than those of radish.

Earlier reports on the development of carotenoids on illumination of etiolated leaves are somewhat inconsistent. Seybold and Egle (1938) reported that the xanthophylls develop more rapidly than the carotenes, whilst Blaauw-Jansen, Kamen and Thomas (1950) reported that the carotenes develop more rapidly. Kay and Phinney (1956) examined the effect of illumination on etiolated maize seedlings and concluded that the observed increase in carotenoids is almost solely due to an increase in the carotenes present, but Goodwin (1958) found that both the carotenes and the xanthophylls exhibited a similar increase on illumination of etiolated maize. Goodwin (1958) also suggests that the development of carotenoids on illumination of etiolated tissue is merely a manifestation of the formation of functional chloroplasts.

In agreement with Goodwin (1958) it was found that the carotenes and the total xanthophylls both increase by a similar amount on illumination, however the relative rates of synthesis do not reflect the concentrations of the respective carotenoids in normal mature leaves; they only serve to produce the normal concentrations. Thus, after about twenty hours, the rates of synthesis of neoxanthin, violaxanthin and β-carotene are somewhat similar, although these carotenoids represent about 13%, 20% and 28% respectively in normal leaves. At the same time, the synthesis of lutein is negligible, although this compound makes up about 40% of the carotenoids in normal leaves.

Very little neoxanthin was present in the etiolated seedlings examined, although Goodwin and Phagpolngarm (1960) report appreciable amounts of this carotenoid in etiolated bean leaves. Neoxanthin was found to be rapidly synthesised on illumination and this is similar to the results found by Krinsky, Gordon and Stern (1964) for the regreening of dark-grown Euglena. No evidence was seen in this work for any relationship between antheraxanthin and zeaxanthin as reported by Krinsky (1964, 1966), although continuous illumination would be expected to favour such changes. Since Krinsky suggests that the antheraxanthin-zeaxanthin couple may act as a protective agent against the photosensitized oxidation of chlorophyll, it is possible that the chlorophyll concentration may not have been high enough for such reactions to

occur.

In addition to the use of TLC for the analysis of the leaf carotenoids, this technique has been used to show the presence of the isoprenoid quinones in leaf lipid extracts. Normal adsorption TLC on silica gel or alumina, (together with the mixture of these, known as alusil) has been used successfully for this purpose but reversed phase techniques, on both TLC and paper, have also proved of great value. Polyamide powder layers have been used for the reversed phase chromatography of biological quinones, by Egger and Kleinig (1965), but both polyamide and polyethylene powder layers have been successfully used in this work, in addition to the more traditional method of impregnation of a silica gel layer with a hydrophobic agent such as liquid paraffin. This latter method however, has produced evidence for a lower isoprenologue of the normal plastoquinone-9, which appears to correspond to plastoquinone-8.

The advantages of both adsorbent and reversed phase TLC have been combined in a novel system of carrying two different materials on a single TLC plate. The best results have been obtained with polyethylene powder layers carrying a 4cm strip, or corridor, of silica gel. The leaf extract is loaded onto the origin of the silica gel strip and development is carried out along the length of the silica gel. After the first development followed by thorough drying, a reversed phase separation may be achieved by turning the

plate through 90° and developing in the second dimension. This system is comparable to the two dimensional paper chromatographic system described by Booth (1962, 1965).

TLC of the isoprenoid quinones has been mainly used for the identification of fractions obtained by the gradient elution chromatographic technique developed in this work. This technique is by no means prefect and several possible improvements can be suggested. Better resolution could probably be obtained using longer, and possibly narrower, columns but this would entail higher back pressures and a suitable pump would have to be found, bearing in mind the difficulties encountered in using piston pumps with the solvents required.

An additional improvement could be made by using two different path-length flow cells in series, together with the necessary optical equipment. This would allow plastoquinone A to be measured directly, as well as the other quinones present at lower concentrations. An additional modification could be the reduction of the quinone by some means in the detection system to enable the difference between the absorption of the quinol and the quinone to be used for the estimations. The use of this difference value would cancel out any difficulties due to other ultra violet absorbing components which may occur in the leaves of some species (e.g. in Gossypium, as reported by Griffiths, Wallwork and Pennock, 1966). A further modification using a suitable dye reagent may allow the tocopherols present in

leaf extracts to be estimated simoultaneously with the quinones.

Although all these suggested modifications could be applied to the assay method, excellent results were obtained with the gradient elution system utilised throughout the majority of this work on the isoprenoid quinones. In view of these results it seems possible that the gradient elution method for the separation and estimation of carotenoids, recently reported by Davies (1967) may prove superior to the TLC system developed and utilised in this present work on carotenoids, provided that sufficient separation of some of the minor carotenoids can be achieved. Davies (1967) makes no mention of this fact but at least two cells of different path lengths would be required to study both the minor and major carotenoids present in any tissue extract.

The gradient elution technique of quinone analysis has been successfully used to study the variation of the phytoquinones, both during the growing season and during the illumination of etiolated leaves. Very few results on the seasonal variation of the phytoquinones can be found in the literature. Hindberg and Dam (1965) reported on the seasonal variation of the bioquinones and α-tocopherol in oak leaves, but very few results are given, although their plastoquinone X, which appears to correspond to plastoquinone B, remains relatively constant. Bucke and Hallaway (1966) describe the seasonal variation of plastoquinone C in young bean plants and report that plastoquinone C is at

a maximum and α-tocopherolquinone at a minimum during the summer. During the winter these workers found high levels of α-tocopherolquinone, whilst plastoquinone C appeared to be absent. Crane, Henninger, Wood and Barr (1966) suggest that all the quinones increase in concentration during the summer, but maintain a fairly constant relationship to each other.

The results of this present work are shown in figs. 71 and 72 in the Results Section and it can be seen in fig. 72 that two quinones remain fairly constant with respect to chlorophyll, at least during the months from February to August. These quinones are phylloquinone and plastoquinone B. This is in agreement with the few results of Hindberg and Dam (1965). The constancy in phylloquinone content was unexpected since Lichtenthaler (1962) has reported that phylloquinone contents vary during the course of the day. Egger (1963) has noted a constant relationship between phylloquinone and chlorophyll a in a number of different species and has suggested that this is an obligate relationship.

The average value of 0.23mg/100mg chlorophyll found for phylloquinone is somewhat low compared with the results of Lichtenthaler and Calvin who find about 1.8 moles/100 moles of chlorophyll (equivalent to 1.0mg/100mg). Similar values to the latter are given by Henninger, Dilley and Crane (1963) although about half of this value was originally

reported by Kegel, Henninger and Crane (1962) for spinach chloroplasts. Hindberg and Dam (1965) however, report the concentration of phylloquinone in normal oak leaves to be between 0.24 and 0.5mg/100mg chlorophyll.

The average concentration of plastoquinone B found is about 0.85mg/100mg chlorophyll as compared with the range of about 0.3mg-9.0mg/100mg chlorophyll calculated from the results of Griffiths, Wallwork and Pennock (1966) for a range of different plants. Crane, Henninger, Wood and Barr (1966) in a review of their group's work, report chlorophyll:quinone ratios of from 40 to 300 in spinach chloroplasts but, from a consideration of Rf values it is possible that some of their work may be on the plastoquinone B-type component found in column fraction IIIA + IV in this present work.

The plastoquinone C concentrations reported by Crane, Henninger, Wood and Barr (1966) correspond to a range of about 1.4-2.4mg/100mg chlorophyll for spinach chloroplasts, as compared with a variation of from 0.75-1.6mg/100mg chlorophyll for the leaves of spinach beet examined in this work. It should be remembered that this value may rise much higher in the late summer, particularly when the chlorophyll concentrations begin to fall. In agreement with Crane et al, the plastoquinone D content was usually between one half and two thirds of the plastoquinone C concentration.

In agreement with the work of Bucke and Hallaway (1966) a seasonal variation in the α -tocopherolquinone content

was observed which exhibited an inverse relationship with the variation in the chlorophylls and in plastoquinones A, C and D. The assay technique described by Bucke and Hallaway (1966) however, was found to be slightly unreliable, in that the fraction containing plastoquinone D and α-tocopherolquinone also contained traces of another compound, at least in etiolated leaves, that exhibited a slight increase in optical density on the addition of potassium borohydride. This may correspond to the "carotenoid-like substance" reported by Crane (1959b), and tends to yield slightly low values for plastoquinone D (or C if this is present). This contaminating component can be recognised in tissues which are devoid of plastoquinone by the ratio of the changes at 262 and 254nm respectively. For pure α-tocopherolquinone, this ratio should be 1 (Bucke and Hallaway, 1966) but ratios higher than 1 are obtained in the presence of this other material. This effect could cause low concentrations of plastoquinone to be missed in the presence of α-tocopherolquinone. Thus the absence of plastoquinone C during the winter, reported by Bucke and Hallaway (1966) may possibly be due to this interfering substance, or alternatively the light intensity available or the age of the plants used may not have been sufficient for the development of plastoquinone C, since both plastoquinones C and D could be detected in all the winter-grown plants examined in this

work (these were outdoor-grown plants and not indoor-grown as used by Bucke and Hallaway)

The only published work describing the development of the isoprenoid quinones on illumination of etiolated leaves, consists of the recent reports of Gaunt and Stowe (1967), Threlfall and Griffiths (1967) and Griffiths, Threlfall and Goodwin (1967). In contrast to the results of the above workers, very little synthesis of phylloquinone was observed on illumination of the etiolated radish leaves examined in this present work. It should be remembered however, that great differences may exist in the behaviour of different leaf and cotyledon materials.

Although the behaviour of plastoquinone B and ubiquinone on illumination is rather uncertain, it seems likely that etiolated dicotyledonous leaves contain both of these compounds, together with somewhat lower levels of plastoquinone A. The presence of ubiquinone and plastoquinone A may perhaps be expected from the results of Threlfall and Goodwin (1963) on plant tissue cultures. These workers reported that low levels of terpenoids, including ubiquinone and plastoquinone A, were detectable in non-chlorophyllous plant tissue cultures.

The parallel behaviour of plastoquinone B (if this fraction is indeed plastoquinone B) and phylloquinone on illumination could possibly be predicted from the results of the seasonal variation experiments, where these two

components are both found to remain at a constant level with respect to chlorophyll. The identical behaviour of phylloquinone and plastoquinone 8 in these tissues may perhaps reflect a single site for each, these sites would possibly be very closely connected with chlorophyll (or possibly, merely chlorophyll a). Crane and Henninger (1966) suggest that phylloquinone may be associated only with light reaction 1 ("System 1") of photosynthesis, whilst plastoquinones A and C may operate in both light reactions 1 and 2. The synthesis of α-tocopherolquinone observed on illumination of etiolated radish leaves is similar to that reported by Threlfall and Griffiths (1967) and Griffiths, Threlfall and Goodwin (1967) for etiolated maize and barley shoots.

Barr, Huang and Crane (1966) reported that the increase in plastoquinones A and C in horse chestnut leaves during the summer may be partially associated with an increase in the number and size of the osmiophilic globules present, and Wellburn and Hemming (1967) observed that one third of the plastoquinone A was not associated with the chloroplast in mature (24 week old) horse chestnut leaves; this may be attributable to loss of osmiophilic globules. It seems probable that the increased amounts of the plastoquinones A and C found during the summer may be located in the osmiophilic globules of the chloroplast. In this case the concentrations of all the chloroplast quinones in the lamellae could be expected to remain constant with respect to chloro-

phyll, irrelevant of the time of year. If this is so, the osmiophilic globules should lack both plastoquinone B and phylloquinone under normal circumstances. The case with respect to α-tocopherolquinone is uncertain but, from the results of Lichtenthaler and Calvin (1964), this compound appears to be located in the lamellae.

The situation postulated above would suggest that all the quinones of the photosynthetic apparatus (located in the lamellae of the chloroplast) apart from the α-tocopherol-quinone, would maintain a constant level with respect to chlorophyll, at least in any one species. In this context it would be of interest to see if the carotenoids present also maintain a similar constant level throughout the season. If this were so it may suggest a constancy of the lamellar constitution which could exist throughout a number of different higher plant families, although the absence of plastoquinone B in the monocotyledons must be borne in mind, and such factors as light intensity may modify the normal levels of various components.

SUMMARY

1) The literature covering the fields of isoprenoid quinones, leaf carotenoids and gradient elution chromatography has been comprehensively reviewed, the latter with particular reference to the methods available for the production of concentration gradients. The literature concerning the other lipid components of the chloroplast, together with the role of lipids in photosynthesis, has also been reviewed, although to a lesser extent.

2) A system for the production of complex concentration gradients has been devised, based on the use of a multi-chamber apparatus with the chambers connected in series by means of pumps. With the aid of the Computer Laboratory, the gradients produced by such a system have been examined and some general principles governing such gradients have been discussed.

3) The gradient producing system above has been used in the development of a semi-automatic, gradient elution chromatographic assay method for the plant quinones, and this method has been used to study the variation of these quinones throughout the growing season. This method appears to be more accurate and reproducible than those at present in use, as well as achieving more rapid analysis.

4) Thin layer chromatography has been used, in both adsorbent and reversed phase applications, together with reversed phase paper chromatography, to examine the caroten-

oids and quinones present in leaves. Various other lipids present have also been studied, including the plant chromanols.

5) A thin layer technique for the separation of leaf carotenoids has been developed from 4) above and this, combined with spectrophotometry, has been used as a rapid assay method for the detection and estimation of the leaf carotenoids.

6) The development of carotenoids, chlorophylls and quinones on illumination of etiolated tissue has been examined, using the techniques summarised in 3) and 5) above, together with a standard spectrophotometric assay for the chlorophylls.

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